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Historical genetic diversity of the greater short-tailed bat (*Mystacina robusta*)

A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Master of International Nature Conservation

at

Lincoln University, New Zealand

and

Georg-August-Universität Göttingen, Germany

by

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2020

Abstract of a thesis submitted in partial fulfilment of the requirements for the degree of a Master of International Nature Conservation.

The genetic diversity of the greater short-tailed bat (*Mystacina robusta*)

By

Isobel F. Oldfield

The greater short-tailed bat (*Mystacina robusta*) is considered critically endangered and potentially extinct. It is believed that if this species is extant it is likely to only reside on small islands south of Stewart Island, such as Big South Cape. In addition it is likely that the population size of this species would be severely restricted.

The research presented in this thesis uses *M. robusta* specimens held in the Museum of New Zealand Te Papa Tongarewa, Wellington (Te Papa) to sequence mitochondrial DNA (mtDNA) control region. Samples included bones collected on the mainland of New Zealand that are likely to be over 200 years old, as well as more recent samples from bats collected in the 1950s and 60s.

A relatively new, modified soaking technique was used to extract DNA from the majority of bone samples. This method was used to ensure that the bones would not be damaged. Given the conservation status of *M. robusta* all specimens are considered precious and therefore extensive sampling of museum collections cannot be undertaken using traditional destructive methods. Tissue and fur taken from whole preserved specimens and a number of broken bones were also used to extract DNA using the more common (and destructive) techniques.

DNA sequences were obtained from a total of nine specimens. None of the sequences obtained were able to be definitively confirmed as *M. robusta*.

While one specimen was found to be a misidentified *M. tuberculata* (NMNZ S.334376 Canterbury), four specimens (NMNZ S.34160, NMNZ S.34237, NMNZ S.38824 and NMNZ S.38824B) were found to differ by multiple SNPs from >200 existing *M. tuberculata* reference sequences and therefore are attributed to *M. robusta*. Some SNPs within the *M. robusta* sequences point to possible haplotype variation between geographic regions that may warrant further investigation. This study demonstrates the potential for

aDNA to be retrieved from *M. robusta* bones in a non-destructive way for molecular analysis to shed new light on the past distribution and population structure of this species.

Acknowledgments

I would like to thank my supervisors Adrian Paterson and Jamie Wood for their help, guidance and quick responses to my questions. I would also like to thank Te Papa Tongarewa (Museum of New Zealand) for allowing me to extract DNA for their museum specimens and for the use of their laboratories. I would particularly like to thank Lara Shepherd from Te Papa for her help with identifying specimens and undertaking laboratory work.

I would like to thank my partner Jason Croucher for the many cups of tea and patience and finally I would like to dedicate this thesis in memory of my father, Stephen Oldfield, who believed so much in the importance of further education.

Contents

Chapter One – Introduction

1.1 Background

As with much of New Zealand's wildlife, the native mammals of New Zealand reflect its long isolation. While the marine environment includes an abundance of native and endemic mammals, for several million years before human arrival, the only terrestrial mammals were New Zealand's bat species (Gibb & Flux, 1973; King, 2005). The introduction of a range of terrestrial mammals has had a significant negative effect on the New Zealand environment (King, 2005). The first mammals to be introduced were brought by Polynesian settlers in the 13th Century AD, the kiore or Pacific rat (*Rattus exulans*) and the kurī or Polynesian domestic dog (*Canis lupus familiaris*). The second wave of introduced mammals were brought to New Zealand by European colonists, who over time introduced a total of 54 mammalian species (King, 2005).

The arrival of people and the introduction of many exotic mammalian species had a devastating impact on the New Zealand environment. During the last 750 years, forest cover has been reduced from an estimated 78% to 23% and 31 alien mammalian species have become established (Hand, et al., 2009). New Zealand has lost a total of 53 native bird species as a result of humans arriving in New Zealand. Approximately 15 of these bird species have become extinct since 1850 (Miskelly, 2015).

New Zealand's terrestrial mammals did not escape the effects of these introductions. The lesser short-tailed bat (*Mystacina tuberculata*) population is estimated to have reduced from 12.5 million pre-humans to approximately 50,000 today (Hand, et al., 2009; Meduna, 2007). The last confirmed sighting of its closest relative, the greater short-tailed bat (*M. robusta*), was in 1965 (Bell, Bell, & Merton, 2016; Hill & Daniel, 1985; Lloyd B. D., 2005)

1.2 New Zealand bats

The only known land mammals native to New Zealand are three small bat species, the long-tailed bat (*Chalinolobus tuberculatus*), *M. tuberculata* and *M. robusta*. All three species are endemic to New Zealand (Daniel, 1976; Te Papa Tongarewa, n.d.; Worthy, Daniel, & Hill, 1996).

Mystacina robusta and their close relatives, *M. tuberculata*, are the only species from the family Mystacinidae still extant (Dwyer, 1960; Hill & Daniel, 1985; Lloyd B. D., 2005; O'Donnell, Christie,

Hitchmough, Lloyd, & Parsons, 2009). Fossil records, however, indicate that the Mystacinidae was previously much more diverse. Records indicate that at least ten species of mystacinids once existed across New Zealand (six species including *M. tuberculata* and *M. robusta*) and Australia (four species) (Hand S. , 2018). At the St Bathans deposit in Central Otago, four New Zealand bats have been uncovered, including the largest yet found – *Vulcanops jennyworthyae* (weighing about 40 g) and the oldest - *Mystacina miocenalis* (Hand, et al., 2018; Hand S. , 2018).

Similarly to *M. tuberculata* and *M. robusta* the diet of *M. miocenalis* is likely to have been omnivorous, given their similar molar morphology, with no features which would suggest specialisation of the diet (Hand, et al., 2015). In contrast *V. jennyworthyae* had more specialised teeth which suggests a slightly different diet, which is likely to have included a range of diverse plant food as well as small invertebrates (Hand, et al., 2018; Hand S. , 2018).

The other two bat fossils uncovered have yet to be formally described but are thought to be related to modern New Zealand mystacinid (Hand, et al., 2013; Hand, et al., 2018; Hand S. , 2018). All the bat fossils found at St Bathans indicate that a semi-terrestrial bat lineage has evolved in New Zealand for at least 16 million years (Hand, et al., 2013; Hand, et al., 2015; Hand S. , 2018). It is likely that global climate change in the middle of the Miocene, which resulted in significant changes to the environment and vegetation, triggered the significant loss of bat diversity pre-human arrival in New Zealand (Hand, et al., 2018; Hand S. , 2018).

Chalinolobus tuberculatus is found in the North Island and some areas of the South Island, including the smaller outlying islands (Daniel & Williams, 1984; Dwyer, 1962), and is considered 'nationally threatened' (O'Donnell, et al., 2017). *Chalinolobus tuberculatus* is the New Zealand bat species most likely to be observed by the public because it usually roosts and feeds along forest edges (Daniel & Williams, 1984; Dwyer, 1962; Molloy, 1995; Te Papa Tongarewa, n.d.).

Mystacina tuberculata is divided into three sub-species, northern lesser short-tailed bat (*M. t. aupourica*), central lesser short-tailed bat (*M. t. rhyacobi*) and southern (or South Island) lesser short-tailed bat (*M. t. tuberculata*). The three subspecies are classified under the Department of Conservation threat classification system as nationally vulnerable, at risk – declining, and at risk – recovering, respectively (Department of Conservation, n.d.; O'Donnell, et al., 2017). *Mystacina tuberculata* is generally found in native forest where they roost singly or in a group (Department of Conservation, n.d.; Molloy, 1995; Te Papa Tongarewa, n.d.).

Mystacina robusta is endemic to New Zealand and the largest of the three New Zealand bat species (Dwyer, 1960; Hill & Daniel, 1985; O'Donnell, Christie, Hitchmough, Lloyd, & Parsons, 2009). This species is currently listed as data deficient, potentially extinct under the New Zealand threat classification and the IUCN red list (O'Donnell C. , 2008; O'Donnell, et al., 2017).

1.2.1 Mystacinidae

Mystacina robusta was initially identified as a subspecies of *M. tuberculata* (*M. t. robusta*) (Dwyer, 1962), however these bats were subsequently confirmed as a distinct species in 1985 (Hill & Daniel, 1985), nearly 20 years after the last confirmed species sighting. In general, *M. robusta* is larger than *M. tuberculata*, with the extremities being relatively shorter. There appears to be few other clear morphological features that can be used to separate the two species (Dwyer, 1962; Hill & Daniel, 1985; Lloyd B. D., 2005).

Mystacinids spend an unusually large proportion of their time on the ground. They furl their wings and use their strong hind legs to burrow through the leaf litter in search of insects (Daniel, 1979; Department of Conservation, n.d.; Hand, et al., 2009; TERRAIN, 2018; TerraNature Trust, 2013). This adaptation also allows these bats to take flight from level ground easily, leaping into the air before flying almost vertically upward (Dwyer, 1962; Lloyd, 2001). Mystacinids are the only bats in the world equipped to forage for food on the forest floor (Department of Conservation, n.d.; Hand, et al., 2009), which likely makes these bats particularly vulnerable to introduced predators, such as rats and feral cats (Daniel & Williams, 1984; Molloy, 1995; Zoological Society of London, n.d.). Lloyd (2001), however, considered that these bats would not be easy prey while on the forest floor as they are cryptic, fast moving, with an acute sense of hearing and smell and who can take flight easily and quickly.

New Zealand mystacinids are thought to have developed a greater degree of terrestrial capabilities compared to other bats due to the absence of mammalian predators in New Zealand, a factor which has also been attributed to the large proportion of New Zealand's endemic bird species that became flightless (Daniel, 1976; Daniel, 1979). A recent study of the remains of an early Miocene mystacinid from Australia (*Icarops aenae*) indicates that this bat was also terrestrially adapted despite coexisting with a number of ground-based mammalian predators. This indicates that mystacinids were already adapted for terrestrial locomotion prior to their isolation in New Zealand (Hand, et al., 2009). It is hypothesised that the adaptions that allowed terrestrial movement may have supported the bat's diverse diet, including the consumption of fruit and nectar within lowland forest (Daniel, 1976; Hand, et al., 2009).

Mystacina robusta was initially widespread across New Zealand and was found throughout the North and South Islands (Hill & Daniel, 1985; Lloyd B. D., 2005; Molloy, 1995; TerraNature Trust, 2013). *Mystacina robusta* disappeared from the New Zealand mainland before European arrival (Daniel & Williams, 1984; Lloyd B. D., 2001; Lloyd B. D., 2005; Worthy, Daniel, & Hill, 1996) and was restricted to small predator-free islands south of Stewart Island, such as Big South Cape (Taukihepa) and Solomon (Rerewhakaupoko) Islands, until rats were accidentally introduced in 1963. There have been no confirmed sightings of *M. robusta* since 1965 (Bell, Bell, & Merton, 2016; Hill & Daniel, 1985; Lloyd B. D., 2005). Invasive predators are a key threat to this species (Lloyd B. D., 2005; Zoological Society of London, n.d.). There is a close association with the appearance of kiore within laughing owl (*Sceloglaux albifacies*) middens and the disappearance of *M. robusta* from the mainland (Lloyd, 2001; Molloy, 1995). It is considered likely that *M. robusta* was more vulnerable to predation by kiore than *M. tuberculata* due to their larger size, as rats would have been able to access crevices used by this bat for hibernation (Lloyd B. D., 2001; Lloyd B. D., 2005).

Mystacina robusta is thought to have been sympatric with *M. tuberculata* on the last few islands they were confined to by the time Europeans arrived; Big South Cape and Solomon Islands (Daniel & Williams, 1984; O'Donnell C. , 1999). This assertion is considered controversial, however, given that *M. robusta* was not considered a distinct species until well after the last sighting of this bat. All specimens collected from Big South Cape, and held at Museum of New Zealand Te Papa, have been confirmed as *M. robusta*, including one juvenile which was incorrectly labelled as *M. tuberculata*. Other collections from the muttonbird islands include specimens labelled as *M. tuberculata*, including two females reportedly collected from Solomon Island (Lloyd B. D., 2005). The bats were known to roost in Puwai Cave on the south coast of Big South Cape and two sea caves at Bat's cave landing on the south east coast of Solomon (McClelland P. , 2009; O'Donnell C. , 1999).

1.2.2 Muttonbird Islands

Big South Cape Island is located approximately 1.5 kilometres south west of Stewart Island, while Solomon Island is located 250 metres north of Big South Cape. The islands are part of a group of 36 islands collectively called Tītī (or muttonbird) Islands where Rakiura Māori have customary rights to collect muttonbirds. The Rakiura Tītī Islands Administering Body manage 18 islands that were formerly owned by the crown. The remaining 18 islands are privately owned (Ballance, 2017). The islands have no permanent inhabitants but are visited every year by muttonbirders for six to eight weeks between April and May (Atkinson & Bell, 1973; Bell, Bell, & Merton, 2016).

Ship rats (*Rattus rattus*) were accidentally introduced to Big South Cape and adjacent islands between 1955 and 1963 and by 1967 *M. robusta* had disappeared. In 1955 an initial incursion occurred with one rat trapped on Big South Cape Island, however, no further rats were detected in subsequent visits in 1956 and 1961 and therefore this was considered a stand-alone event. In 1964, however, muttonbirders returned to find the island infested with rats. The muttonbirders found evidence of the rats everywhere, including within their huts where they had chewed food, supplies, the bedding, and the wallpaper off the walls (Atkinson & Bell, 1973; Bell B. D., 1976; Bell, Bell, & Merton, 2016; O'Connor, 2016). The rats are thought to have been introduced by boat, with DNA analysis has indicated that they likely originated from areas further afield than the nearest land masses of Stewart Island and the south of the South Island (Bell, Bell, & Merton, 2016; Robins, Miller, Russell, Harper, & Fewster, 2016).

In 1964 a wildlife rescue attempt was launched to relocate indigenous birds from rat-infested Big South Cape Island to rat-free islands. South Island saddlebacks (*Philesturnus carunculatus carunculatus*), Stewart Island snipe (*Coenocorypha aucklandica iredalei*) and Stead's bush wren (*Xenicus longipes variabilis*) were all transferred but, with the exception of the South Island saddleback, the transfers were unsuccessful (Bell B. D., 1976; Bell, Bell, & Merton, 2016; Dowding & Murphy, 1994; Miskelly, 2012) and the Stewart Island snipe and Stead's bush wren subsequently became extinct (Bell, Bell, & Merton, 2016; BirdLife International, 2016a; BirdLife International, 2016b). During this salvage attempt abundant numbers of bats in a 'bat cave' were noted and two individuals were mist-netted. At this time, however, *M. robusta* was not recognised as a distinct species and as a result they were assumed to be *M. tuberculata* and no attempt was made to relocate them (Atkinson & Bell, 1973; Bell, Bell, & Merton, 2016).

As a result of this rat incursion event, for the first time, rats were definitively recognised as the cause of extinction of native land birds (Atkinson & Bell, 1973; Bell, Bell, & Merton, 2016). Rats were eventually eradicated from Big South Cape and Solomon Island in 2006 using funding from the Command Oil Spill Trustee Council (McClelland P. , 2009; McClelland, et al., 2011; O'Connor, 2016).

Putauhinu Island, forming part of the southern Tītī Islands, is located approximately 1.5 kilometres north west of Big South Cape Island, and approximately five kilometres south west of Stewart Island. Rats were eradicated from Patauhinu Island in 1997 (Ballance, 2017; McClelland P. , 2009). Prior to 1998 there was no evidence that bats resided on Putauhinu. An Otago University Zoology team visited the island in 1998 and 1999 and reported that a bat was observed on each visit (O'Donnell C. , 1999). In 1999 the Department of Conservation undertook surveys for bats on Putauhinu Island using automatic

bat detectors, mist netting and searches of tree cavities. During the survey two *Mystacina*-like calls were detected at 25 kilohertz within rata forest in the central island area. No calls were recorded at 28 kilohertz (*M. tuberculata* peak frequency) or 40 kilohertz (*C. tuberculatus* frequency). No bats were caught during the 41 hours of mist netting over three nights (O'Donnell C. , 1999). O'Donnell (1999) stated that while confirmation is still required, there is a good chance that the calls heard were those of *M. robusta*. O'Donnell (1999) further noted that the low number of confirmed calls and lack of bats caught imply that if bats were present that their numbers were very low.

Following the eradication of rats from Big South Cape in 2006, a muttonbirder, who had previous experience with bats, reported seeing a bat's head emerge from a tree crevice on the west side of the island. This sighting was reinforced by another sighting of a bat in flight over Murderer's Cove. Researchers returned to Putauhinu and also visited Big South Cape in 2009 to confirm the presence of bats on the islands and, if the bats were found, identify the species (McClelland P. , 2009). The researchers set up 13 automatic bat detectors, four harp traps and three mist nets. One bat was recorded on a hand-held detector and one bat pass was recorded on an automatic detector. No bats were caught in any of the traps deployed. The lack of success indicates that bat numbers on the island were still very low (McClelland P. , 2009).

On Big South Cape automatic bat detectors were set-up along with six harp traps. Mist netting and searching for suitable roost holes was also undertaken although no signs of bats were observed. A number of bat-like calls were detected on three of the automatic bat detectors although these were subsequently attributed to mottled petrel taking off near the bat detectors. At the conclusion of the visit no bats had been caught or positively detected (McClelland P. , 2009).

Overall, it was noted that it is possible that one population of bats exists that moves between the islands. It was considered that management of the islands was unlikely to change even if *M. robusta* are confirmed to be present and, therefore, further attempts to find and identify the bats was not recommended (McClelland P. , 2009).

1.2.3 Ecology

If *M. robusta* is still extant, the remaining population is thought to consist of less than 50 individuals. Very little is known about the ecology and habitat preferences of *M. robusta* (Zoological Society of London, n.d.). *Mystacina robusta* has been recorded roosting in caves and is likely to have also roosted within tree cavities (Daniel & Williams, 1984; Lloyd B. D., 2001; Lloyd B. D., 2005; Stead, 1937; Zoological Society of London, n.d.). Evidence suggests that this bat may have also been found roosting in

abandoned seabird burrows, including those of the sooty shearwater (*Puffinus griseus*) (Daniel, 1979; Daniel & Williams, 1984; Lloyd B. D., 2005).

Mystacina robusta's diet is likely to have consisted of invertebrates taken on the wing and from leaf litter, as well as pollen, nectar and fruit, essentially similar to the diet of *M. tuberculata* (Daniel, 1976; Daniel, 1979; Daniel & Williams, 1984; Lloyd B. D., 2001; Lloyd B. D., 2005). There are also some records which indicate that *M. robusta* may have been carnivorous. Unconfirmed reports stated that these bats may have preyed on muttonbird chicks in the nest and eaten the meat of muttonbirds that had been left hanging out to dry (Daniel, 1979; Dwyer, 1960; Lloyd B. D., 2005; TerraNature Trust, 2013).

Stead (1937) spent five days on Solomon Island in 1931 and caught seven bats he identified as short-tailed bats (note that in Stead's time *M. robusta* and *M. tuberculata* had not been identified as different species). Stead kept these bats for five days and offered a range of food during this time. The only item of food eaten during the five days was the body of a diving petrel which was skinned and left in the cage. Stead noted that the following morning some of the flesh appeared to have been eaten. Lloyd (2001) states that it is likely that all reports of carnivorous activity are related to *M. robusta* as captive *M. tuberculata* could not be induced to consume meat even when it was offered on multiple occasions.

Middens and deposits provide evidence that suggests that native predators of *M. robusta* are likely to have included the now extinct laughing owl (*Ninox albifacies*), morepork (*Ninox novaeseelandiae*) and New Zealand falcon (*Falco novaseelandiae*) (Lloyd B. D., 2001; Lloyd B. D., 2005; Worthy & Holdaway, 1996; Worthy & Zhao, 2006).

A number of estimates have been produced for the mass of *M. robusta*. One estimate indicates that *M. robusta* is likely to have weighed approximately 25-35 g, compared to *M. tuberculata* that weighs approximately 12-15 g (Daniel, 1979; Daniel & Williams, 1984). Another study found that the mass of *M. robusta* is more likely to have been around 14.5 g (Lloyd, 2001). Lloyd (2005), however, indicated that the above calculations may be incorrect due to the use of the forearm length of *M. tuberculata* in the calculations that is now known to be proportionally shorter than that of *M. robusta*. Instead it is hypothesised that because the overall dimensions of *M. robusta* are 20% greater, the linear relationship between dimension and mass would suggest that *M. robusta* was up to 70% heavier, or approximately 24 g (Lloyd B. D., 2005).

A significant increase in size (10-20%) has been observed in *M. robusta* specimens moving from the most southern specimens to the most northerly (Lloyd B. D., 2005; Worthy, Daniel, & Hill, 1996). Bergmann's

law states that the size of homeothermic animals increase along a gradient from warm to cold temperatures, due to the fact that a large body loses proportionally less heat (Oxford University Press, 2020; Worthy, Daniel, & Hill, 1996). In contrast *M. robusta's* size increases along a gradient of cold to warm, which is at odds with Bergmann's law. It has been hypothesised that a smaller size in the south was advantageous for this species of bat to reduce energy expenditure and a larger size in the north was advantageous to facilitate feeding on abundant macroinvertebrates (Lloyd, 2001; Worthy, Daniel, & Hill, 1996). Worthy, Daniel & Hill (1996) considered it likely that *M. robusta* spent more time foraging on the ground than its closest relative, *M. tuberculata*, although no reason for this statement is given. In contrast Lloyd (2001) stated that there is no evidence that *M. robusta* were more terrestrial than *M. tuberculata*. Given the size difference between *M. tuberculata* and *M. robusta* it is considered that *M. robusta* would most likely have eaten larger invertebrates (Worthy, Daniel, & Hill, 1996).

The frequency of maximum intensity of echolocation calls in microbats can be estimated from forearm length. Based on this measurement it is estimated that the maximum intensity of *M. robusta's* echolocation call is one to two kilohertz lower than *M. tuberculata* and is likely to be 26-27 kilohertz (Lloyd B. D., 2001; Lloyd B. D., 2005; O'Donnell C. , 1999; Worthy, Daniel, & Hill, 1996).

Mystacina robusta are likely to have used torpor, similarly to *M. tuberculata* (Lloyd, 2001). In particular, Stead (1937) described removing cold and sluggish bats from a roost during the day in early summer. It is likely that these bats also used seasonal hibernation, making only occasional flights during the winter months (Daniel, 1979; Lloyd B. D., 2001; Lloyd B. D., 2005).

1.2.4 Distribution

Current *M. tuberculata* populations are restricted to extensive areas of undisturbed, old-growth, temperate, closed evergreen forest with low numbers also occurring in areas adjacent to old-growth forest (Hand, et al., 2015; Lloyd, 2001; Sedgeley, 2003). It is estimated that *M. tuberculata* now occupies only a third of its original range (Hand, et al., 2015). The habitat preferences of *M. robusta* are less well known, however it is likely that the preferred habitat type would have been similar to *M. tuberculata* (Hand, et al., 2015). *Mystacina tuberculata* are considered to once have been widespread across both the North and South Island of New Zealand (Lloyd, 2001). It is likely that prior to human arrival *M. robusta* and *M. tuberculata* would have had a similar distribution (Hand, et al., 2015). *Mystacina robusta* fossils have been found in Waitomo, Hawkes Bay and Wairarapa within the North Island and Nelson, Westland, Canterbury and Central Otago within the South Island (Lloyd, 2001).

1.2.5 Aims and Hypotheses

Historical populations and the extent of genetic variation can now be estimated provided there are appropriate sources of ancient DNA. *Mystacina robusta* bones, which can provide a useful source of DNA, can potentially inform us about the extent of population size in the past. Given that *M. robusta* is potentially extinct and has not been sighted since the late 1960s DNA from museum specimens is one of the only new sources of information available for this species.

We know virtually nothing about the genetics of *M. robusta*, including its genetic diversity, phylogenetic relationships or population structure. Ancient DNA offers the potential to begin gaining such information.

In this study I aim to generate the first DNA sequences from *M. robusta*, assess its genetic distinctiveness from *M. tuberculata*, and using a number of individuals to look at whether any potential population structure existed.

Hypotheses for this study include:

- *M. robusta* is a distinct species;
- regional population structure will be evident based on geographic features that may prove barriers to *M. robusta* dispersal; and
- the soaking method will work as an extraction technique.

Chapter Two – Ancient DNA Analysis

Ancient DNA (aDNA) analysis generally involves the extraction of DNA from specimens that were not collected for the purpose of DNA analysis and may include archeological and natural history specimens, which can be hundreds, or sometimes thousands, of years old (Mulligan, 2005; Paabo, et al., 2004). aDNA can be extracted from museum skins, skeletal material, archaeological material or paleontological remains. Natural processes result in the degradation of DNA, which makes aDNA much lower quality than modern genetic material (Leonard, 2008; Paabo, et al., 2004).

DNA begins to degrade as soon as an organism dies due to break down by enzymes, bacteria, fungi and insect interactions (Linderholm, 2016; Paabo, et al., 2004; Shapiro & Hofreiter, 2014). As it degrades, DNA breaks into fragments, which means the longer the organism has been dead the shorter the DNA fragments become (Ho & Gilbert, 2010; Linderholm, 2016; McKie, 2014; Morris, 2015). DNA degradation leads to three problems, reduced quantity of DNA, smaller fragments and DNA damage that can lead to identification of false mutations (Ho & Gilbert, 2010; Leonard, 2008; Paabo, et al., 2004).

In ancient samples, there is much less DNA available than from fresh samples. Sources of modern DNA are pervasive in the surrounding environment. Contamination or extraneous DNA is a significant problem for aDNA studies and must be managed carefully (Ho & Gilbert, 2010; Leonard, 2008; Linderholm, 2016; Shapiro & Hofreiter, 2014; Slatkin & Racimo, 2016). This issue is particularly true of studies involving ancient humans, as modern human DNA is ubiquitous within museums and laboratories (Hofreiter, Serre, Poinar, Kuch, & Paabo, 2001; Paabo, et al., 2004). Sequencing DNA for ancient, and in particular, extinct animal species, such as *M. robusta*, poses less of a problem because the identification of distinct sequences which relate to extant species in the same group will provide confirmation of reliable results (Hofreiter, Serre, Poinar, Kuch, & Paabo, 2001).

The rate of decomposition of DNA is dependent on environmental factors, such as temperature, burial or storage conditions and the number of microbes present (Alex, 2017; Linderholm, 2016; Shapiro & Hofreiter, 2014). As a result, methods for extracting and amplifying aDNA have to be adjusted. Poinar (2000) noted that the field presents extreme difficulties due to the minute amount and degraded nature of the target species DNA and the exceptional risk of contamination. Methods used to minimise the risk of cross contamination must therefore be rigorous and findings carefully scrutinised.

Assuming standard conditions, it is often estimated that it would take 100,000 years for all DNA in a specimen to be destroyed (Hofreiter, Serre, Poinar, Kuch, & Paabo, 2001). A study of the half-life of DNA in bone using 158 dated Moa (*Dinornithiformes*) bones found that DNA decay can be described using first-order kinetics. The study authors predicted that in the right environment DNA fragments could still be present one million years after deposition (Allentoft, et al., 2012). Various environmental factors will increase or decrease the timeframe over which DNA will be destroyed, although extracting DNA sequences from a specimen more than one million years old is unlikely to be possible (Hofreiter, Serre, Poinar, Kuch, & Paabo, 2001; Ho & Gilbert, 2010; Paabo, et al., 2004). Most studies use DNA that is less than 50,000 years old and many of the older specimens come from cold climates (Alex, 2017). To date sequences have been successfully extracted from bones recovered from permafrost that are 700,000 years old, and greater than 300,000 years old from bones outside permafrost (Dabney, et al., 2013; Hagelberg, Hofreiter, & Keyser, 2015; Shapiro & Hofreiter, 2014).

Polymerase chain reaction is a method used to make copies of a target DNA sequence. The required sequence is targeted using a primer (short DNA fragment complementary to the target DNA) and that sequence is then exponentially amplified to generate thousands to millions of copies (Garibyan & Avashia, 2013). Progress in aDNA gained substantial traction with the development of PCR that enables the amplification of very small amounts of DNA (Garibyan & Avashia, 2013; Ho & Gilbert, 2010; Orlando & Copper, 2014; Paabo, et al., 2004). Over the past decade a variety of new techniques for creating ancient DNA libraries, enriching for endogenous DNA and high throughput sequencing have vastly increased the potential for gaining genetic information from marginal specimens. However, PCR and Sanger sequencing approaches still have a place for particular specimens and research questions.

The degraded and fragmented nature of aDNA means numerous polymerase chain reactions (PCRs) are required to sequence a complete mitogenome. This can lead to repeated destructive sampling of ancient and often invaluable specimens (Ho & Gilbert, 2010). Despite these barriers a number of complete mitochondrial genomes have been sequenced, including two extinct moa (*Emeus crassus* and *Dinornis giganteus*) (Cooper, et al., 2001), a Pleistocene mammoth (*Mammuthus primigenius*) (Rogaev, et al., 2006), and a middle Pleistocene cave bear (*Ursus deningeri*) (Dabney, et al., 2013). The sequencing of the complete mitochondrial genome of the two moa species was used to demonstrate that the moa and kiwi are not a monophyletic group as was previously thought but rather represent two separate migrations to New Zealand (Cooper, et al., 2001; Paabo, et al., 2004).

Most studies to date have focused on mitochondrial DNA because there are hundreds to thousands of mitochondria in every cell, while there is only a single nucleus. This means that the chances of mitochondrial DNA surviving in an ancient sample is much greater than for nuclear DNA (Orlando & Copper, 2014; Paabo, et al., 2004).

The first study using what became known as aDNA was conducted in 1984 when 229 base pairs from a 140-year-old Quagga museum specimen were successfully sequenced (Alex, 2017; Higuchi, Bowman, Freiberger, Ryder, & Wilson, 1984; Ho & Gilbert, 2010; Linderholm, 2016; Orlando & Copper, 2014). This study was the also the first study to demonstrate that DNA could be extracted from an extinct species (Higuchi, Bowman, Freiberger, Ryder, & Wilson, 1984). The following year this process was replicated when ancient human DNA was recovered from an Egyptian mummy several thousand years old (Ho & Gilbert, 2010; Linderholm, 2016; Paabo, 1985; Orlando & Copper, 2014).

Further studies followed, including one that claimed to have retrieved DNA from an 80-million-year-old dinosaur bone (Woodward, Weyand, & Bunnell, 1994). This claim was subsequently found to be most likely the result of contamination because the finding could not be replicated (Hedges & Schweitzer, 1995; Linderholm, 2016). As a result of some of these early questionable findings, more rigorous controls and standards were adopted to provide more confidence in results (Linderholm, 2016; Cooper & Poinar, 2000).

It is now standard practice to extract aDNA in clean-room conditions, which may include bleach treatment of surfaces and the use of filtered air systems. These dedicated aDNA laboratories are kept separate from those laboratories used for the extraction of modern DNA (Hofreiter, Serre, Poinar, Kuch, & Paabo, 2001; Paabo, et al., 2004; Slatkin & Racimo, 2016).

In 2010, researchers used DNA from a finger bone to sequence the genome of what turned out to be a new archaic hominin group. This is the first such group to be characterised entirely from DNA sequencing as the fossil remains found were too scarce to provide sufficient morphological information (Hagelberg, Hofreiter, & Keyser, 2015; Reich, et al., 2010; Slatkin & Racimo, 2016).

Threatened species management and conservation can be aided through the analysis of aDNA by connecting observations between modern and ancient populations that can help identify how populations have shifted or changed as a result of human interactions. This is particularly important for species with few morphological differences that may be difficult to separate by visual observations,

(Rawlence, et al., 2017; Rodrigues, McKechnie, & Yang, 2018) or for highly mobile species where breeding locations may not be able to be determined (Eda, et al., 2012).

Many endangered species have small populations and restricted ranges and DNA analysis that focuses on modern samples can introduce bias when estimating population parameters. The analysis of aDNA provides an opportunity to remove assumptions and help develop historic estimates of population size, level of gene flow and to identify relationships with other populations. Understanding historical population parameters are critical in setting conservation and management goals for threatened species (Leonard, 2008; Orlando & Copper, 2014). In addition aDNA provides an opportunity to study human-environment relations from the past with the view of determining how human impacts may impact biodiversity into the future (Hofman, Rick, Fleischer, & Maldonado, 2015).

There are many aDNA applications that can aid conservation management, some of these include:

1. helping to identify which species to protect;
2. identifying sub-species that can be reintroduced to historic ranges;
3. assisting with re-introductions from captive breeding programmes;
4. assisting in setting conservation targets through identifying historic population sizes;
5. managing population bottlenecks;
6. evaluating human impacts on populations; and
7. providing key information on how ecosystems' respond to long-term factors, such as climate change and disease (Hofman, Rick, Fleischer, & Maldonado, 2015; Leonard, 2008).

Controversially, aDNA techniques could also provide an avenue for 'de-extinction' through the use of the aDNA from extinct specimens, meaning that in the future extinction may not be a permanent state (Shapiro & Hofreiter, 2014).

Examples which reflect the conservation management applications identified above are provided below.

1. Identifying true distinct species as opposed to an aberrant morphology of an existing species or rare hybrid is important for determining whether such a species warrants protection (Leonard, 2008). The large-billed reed warbler (*Acrocephalus orinus*) is only known from the type specimen collected in 1867. DNA evidence indicated that the specimen was representative of a species distinct from the closely related *A. dumetorum*. It is considered that this species may still exist in the wild and may have been overlooked due to some references that this species is synonymous with *A. stentoreus* (Bensch & Pearson, 2002).

2. The historic population structure of the short-tailed albatross (*Phoebastria albatrus*) was determined using aDNA obtained from archaeological bones. This study identified that the albatross present within two breeding regions (Torishima in the Izu Islands and two islets of the Senkaku Islands) are descended from two ancient populations, despite no sub-species being proposed for the short-tailed albatross. This has implications for conservation, given that one of the breeding sites now has drastically reduced genetic diversity (Eda, et al., 2012).
3. Smulders et al. (2003) studied the genetic diversity of a Dutch population of common hamsters (*Cricetus cricetus*) with the aim of answering two questions: do the animals belong to a separate subspecies, and did they experience a severe loss of genetic diversity? DNA analysis of current populations and museum samples enabled the study to confirm that the Dutch population of common hamsters was not a subspecies but was highly constrained genetically. This means that animals from other populations can be introduced to the existing captive breeding programme to improve genetic diversity of the existing animals. The red wolf (*Canis rufus*) is considered endangered and was rescued from extinction in the wild by captive breeding. A study of captive bred red wolves found that those animals accurately reflect the composition of the pre-1940 wild population and that because those animals once lived in the wild, the captive population can justifiably be used for reintroductions (Roy, Geffen, Smith, & Wayne, 1996).
4. Grey wolves (*Canis lupus*) have disappeared from much of the United States. DNA analysis has indicated that the historic North American wolves were likely a subset of the still existing Canadian populations. This indicates that the historic US population could be restored from natural or planned reintroductions from Canada. The study also found that Mexican grey wolves could also be introduced to North America to mimic past intergradation. The study authors concluded the planned reintroductions should be guided by ecology rather than genetic heritage (Leonard, Vila, & Wayne, 2005).
5. A study of recent and museum samples of pumas (*Puma concolor*) indicated that the entire North American population (186 individuals from 15 previously named sub-populations) was genetically homogeneous. The previous classification of 32 subspecies of puma was not confirmed through DNA analysis, with six broad phylogeographic regions identified instead (Culver, Johnson, Pecon-Slattery, & O'Brien, 2000). A study of Yellowstone grizzly bears (*Urus arctos*) indicated that, despite previous reports the population does not appear to have undergone a bottleneck and genetic diversity was higher than previously thought. The authors

commented that this study shows the importance of historical specimens and genetic analysis for conservation management (Miller & Waits, 2003).

6. Genetic diversity has been shown to be low in New Zealand snapper (*Pagrus auratus*) populations as determined using aDNA techniques, with the research indicating that there has been a significant decline in diversity of the population since human exploitation began (Hauser, Adcock, Smith, Ramirez, & Carvalho, 2002). Traditional population theory suggests that genetic diversity will only be severely restricted in populations that are small in size. The study of New Zealand snapper suggests that this may not be the case and that over-exploitation can result in low genetic diversity even when population size remains relatively large (Hauser, Adcock, Smith, Ramirez, & Carvalho, 2002).
7. Barnes et al. (2002) used DNA preserved in permafrost remains to study the genetic changes in the brown bear (*Ursus arctos*) as a result of the climatic and environmental changes associated with the last glaciation. Studies such as this may provide guidance for conservation managers in the face of human induced climate change (Leonard, 2008).

A picture of past environments can also be built through the analysis of aDNA from sediment cores, which provide a picture of the fauna and flora that were present in the region at various times (Paabo, et al., 2004; Parducci, et al., 2017; Willerslev, et al., 2003). It is noted however that dating these samples remains challenging when DNA can move between layers, for example due to the percolation of water (Paabo, et al., 2004; Rawlence, et al., 2014). This method may, however, provide a way for researchers to avoid more destructive methods of sampling (Willerslev, et al., 2003). Similarly, coprolites (fossilised faeces) can provide information regarding habitat and dietary preferences in the environment at the time the coprolite was deposited (Wood, et al., 2013).

Chapter Three – Methods

3.1 Specimen Identification

A number of museums across New Zealand were approached to determine the size of their *M. robusta* collections and to determine whether sampling any of their collection would be possible. Records were provided for three museums, Auckland War Memorial Museum, Canterbury Museum and the Museum of New Zealand Te Papa Tongarewa, Wellington (Te Papa). The Te Papa collection appears to be the most extensive collection of *M. robusta* specimens and a request was placed with Te Papa to sample a number of the specimens held in their collection.

Samples were selected based on location data. A spreadsheet of all specimens held at Te Papa was provided. Specimens were selected that had associated map coordinates and covered as many localities across the New Zealand mainland as possible and a request to sample these specimens was made. In addition a request was made to take samples from more recent bat bone and pelt specimens, which were labelled as being collected from Stewart Island.

It should be noted that these 'Stewart Island' specimens were collected in 1951 and the location indicated may not be accurate. It is considered likely that neither *M. tuberculata* nor *M. robusta* were ever collected from Stewart Island, although it is likely they both occurred there. Specimens labelled as having originated from Stewart Island are likely to have come from one or other of the surrounding islands, such as Big South Cape or Solomon Islands (Hill & Daniel, 1985; Daniel & Williams, 1984).

Mystacina robusta are expected to have been extinct from mainland New Zealand by the time Europeans had settled New Zealand, approximately 200 years ago. This means that any bone specimens found in cave deposits within the mainland are likely to be older than 200 years. In contrast *M. robusta* are known to have existed on islands, south of Stewart Island until the 1960s. For the purpose of this thesis it has been assumed that all bat bones collected from mainland New Zealand are older than 200 years and any preserved bats, or bat pelts labelled as collected from Stewart Island in 1951 were from the population of bats from offshore islands that was still extant at the time.

A list of the Te Papa specimens requested for sampling and a map of the locations of those specimens is provided in Appendix A. Permission was given for a subset of the specimens requested to be sampled. Reasons given for declined permission to sample some of the specimens on the list included:

- Repeat of the location of other samples already provided for sampling;
- Precious specimens, such as a single bone;
- Bones considered too small; and
- Specimens sourced from dunes, and therefore considered unlikely to contain preserved DNA because dune sites are typically poor preservers of DNA.

In total permission was granted for specimens from 23 registration lots to be sampled. Many of the registration numbers referred to a large collection of bones found in one location, as shown in Figure 1, and in some cases multiple bones were sampled from the same registration number. Three additional registration lots were added, that were not included on the specimen spreadsheet provided by Te Papa (because they were stored in a different collection, that had limited access due to earthquake damage). These were NMNZ LM001513, NMNZ DM1629 and NMNZ LM001891, NMNZ DM1629 (four bats) and NMNZ LM001891 (one bat). All these specimens were preserved in ethanol. These bats were collected in 1963 from Big South Cape Island and 1965 from Solomon Island respectively. These specimens were added because they were more recent specimens that may provide information of the genetic diversity of *M. robusta* just prior to the species disappearing.

Figure 1: M. robusta *bones from the Te Papa fossil collection (Photo credit: Isobel Oldfield, 2017)*

All sampling was undertaken in the ancient and modern DNA labs at Te Papa to ensure the specimens were able to remain within Te Papa premises.

3.2 DNA Extraction

DNA is typically extracted through destructive measures which involves grinding up a piece of bone or using blood, hair or tissue. This destructive process makes sampling ancient samples of small boned animals challenging without destroying a precious specimen.

To ensure that the bones that were to be sampled as part of this study were not damaged a modified DNA soaking method described by Tennyson, Cooper & Shepherd (2015) was used to extract DNA from the majority of the specimens. Tissue and fur were also collected from a number of specimens as well as a bone fragment from LM001270 to determine whether DNA could be successfully extracted using the more common destructive method (the commercial Qiagen DNeasy Blood and Tissue kit protocol).

The full step-by-step methodology used for soaking DNA from the specimens is outlined in Appendix B. All extractions and polymerase chain reaction (PCR) set-ups were performed in a dedicated ancient DNA laboratory at Te Papa. All PCRs were conducted in a modern DNA laboratory located in a separate Te Papa building. Potential contamination was monitored through the use of extraction negatives and PCR negative controls.

As noted above, a commercial kit (Qiagen DNeasy Blood and Tissue) was used for all DNA extractions with modifications to enable the soaking method. Each bone was soaked in 200 µL of EDTA, 6 µL SDS and 5 µL proteinase-K. The bones in solution were heated at 45^{o} in a water bath for between 15 and 35 minutes. Following extraction all bones were soaked in distilled water for at least as long as they were heated and then allowed to dry at room temperature overnight. After the bones were dry the bones were searched for signs of overt damage, no damage was observed in any of the bones used. Photographs were taken of each sample and, where the soaking method was used, a photograph after soaking was also taken. These photographs are included in Appendix C.

The resultant solution was then added to 200 µL AL Buffer and vortex, 200 µL of 100% ethanol was then added and this solution was then vortexed. The solution was the centrifuged through a Qiagen DNeasy column. The extraction was then washed using the buffers provided in the Qiagen kit, and eluted in a final volume of 40 µL of Buffer AE.

DNA was extracted from the bone and tissue samples using the destructive sampling method outlined under the Qiagen kit tissue protocol, specifically the protocol for tissue. These samples were incubated at 55°C in a water bath. The first set of samples were incubated for 2 hours (samples 'A'), the second set were incubated overnight (samples 'B').

PCR set-ups were completed using 11 µL of MyTaq (Bioline Australia), 10mg/mL bovine serum albumin (BSA) and the each primer (6 µL MyTaq, 0.5 µL of the forward primer, 0.5 µL of the reverse primer, 2.5 µL of BSA and 1.5 µL of water); 1 µL of the DNA solution was then added. PCR products were then run on an 2% agarose gel. Samples generated a band of approximately the right size during electrophoresis were purified by digestion with 1 U shrimp alkaline phosphatase (rSAP, New England Biolabs) and 5 U exonuclease I (EXO, New England Biolabs) at 37°C for 15 minutes, followed by inactivation of the enzymes at 80°C for 15 minutes.

Purified PCR products were then sent to a commercial laboratory for sequencing. Initially samples were sent to the Massey Genome Service at Massey University in Palmerston North for sequencing. Later samples were sent to Macrogen in South Korea for sequencing.

Table 1: Summary of the museum specimens that were sampled, as well as the method used, the type of sample and the number of samples extracted from each specimen lot

Specimen #	Type of Sample	Method	Location	# Samples extracted
NMNZ LM001511	piece of tissue from ribs	Qiagen kit	'Stewart Island'	3
NMNZ LM001512	piece of tissue	Qiagen kit	'Stewart Island'	4
NMNZ LM001513	piece of tissue + fur	Qiagen kit	'Stewart Island'	2
NMNZ LM001270	bone fragment (dry skeleton)	Qiagen kit	Wairarapa	4
NMNZ S.34270	broken jaw	soaking	North Canterbury	2
NMNZ S.32399	broken jaw	soaking	Tasman	1
NMNZ S.32373	broken jaw	soaking	Tasman	1
NMNZ S.38824	broken jaw	soaking	Tasman	2
NMNZ S.34376	bone fragment	soaking	South Canterbury	1
NMNZ S.34160	bone fragment	soaking	South Canterbury	1
NMNZ S.34127	whole bone	soaking	South Canterbury	1
NMNZ S.33918	broken jaw	soaking	South Canterbury	1

Specimen #	Type of Sample	Method	Location	# Samples extracted
NMNZ S.34224	broken jaw	soaking	South Canterbury	1
NMNZ S.32324	broken jaw	soaking	Tasman	1
NMNZ S.33699	broken jaw	soaking	North Canterbury	1
NMNZ S.30161	whole bone	soaking	Tasman	1
NMNZ S.39187	bone fragment	soaking	Tasman	1
NMNZ S.35793	bone fragment	soaking	Hawkes Bay	1
NMNZ S.35495	bone fragment, distal right humerus	soaking	Hawkes Bay	1
NMNZ S.34237	broken humerus	soaking	South Canterbury	1
NMNZ LM001891	tissue	Qiagen kit	Solomon Island	1
NMNZ DM1629[1]	tissue	Qiagen kit	Big South Cape	3

3.3 Primer Design

No previous studies have extracted DNA for *M. robusta* and thus there are no records of sequences on GenBank[2] (as at April 2020). Therefore primers had to be designed based on existing, available sequences for *M. tuberculata*. Generally primers for a previously unstudied species would be designed using at least two closely related species in order to identify conserved sequence regions in which to design primers. However *M. robusta* only has one closely related species.

All *M. tuberculata* control region sequences available on GenBank were exported into the software Geneious R11 (Kearse et al., 2012). The mitochondrial control region (CR) was targeted for this study because of the relatively high degree of intraspecific variation found at this locus. A total of 232 *M. tuberculata* CR sequences were identified and aligned to enable identification of regions with little variation, but with variability between primers. Primers were designed based on these regions and were designed to amplify short DNA fragments to increase the chance of the primers amplifying the fragmented aDNA.

[1] Note four bats preserved in ethanol fall under this specimen number. Three of the four were sampled. One of those specimens (DM1629.1) to be sampled is thought to be a *M. tuberculata*, rather than a *M. robusta* and therefore a sample was taken in the hope of determining whether this was the case.

[2] https://www.ncbi.nlm.nih.gov/genbank/

Two pairs of primers were designed, Myst_346F and Myst_408R and Myst424F and HBat15958R. The first pair of primers (Myst_346F and Myst_408R) were intended to amplify fragments 62 base pairs (bp) long (between the primers amplicons were ~105 bp). The second set of primers (Myst_424F and HBat15958R) were intended to amplify fragments 89 bp long (between primers amplicons were ~130 bp). Figure 2 below shows the primers used against a *M. tuberculata* reference sequence.

Figure 2: Primers used against a reference M. tuberculata *sequence*

It should be noted that the numbers used in the primer names do not exactly match the primer positions (the primers were named at the beginning of the project). In addition, the control region sequences used for the primer naming does not include the binding position for the HBat15958R primer, as the name used in the Bryan Lloyd study that referenced this primer was retained. It is also worth noting that the length of the sequences used in the analyses may not correspond to the amplicon length as low-quality nucleotides at the ends of sequences were trimmed prior to alignment.

All sequences downloaded from GenBank were originally uploaded by Bryan Lloyd (Department of Conservation) and are associated with his study of the demographic history of *M. tuberculata* (Lloyd B. D., 2003). All primers were designed from scratch except the HBat15958R primer which was used by Lloyd in his 2003 study.

A pair of universal mammalian primers, in combination with a human blocking primer, was also used for PCR amplification at the beginning of this study (16Smam1 and 16Smam2 and 16Smam blkhum3 from (Boessenkool, et al., 2012)). This was done in case the primers that were designed for this study did not work. The general mammalian primer used should result in successful sequencing of any mammal

species if DNA has been extracted. A number of the sequences run with the mammalian primer were successful, indicating the soaking method used was successfully able to extract DNA. These sequences were not considered further because sequences were obtained for all bat-specific primers. The sequences obtained from the mammalian primer were therefore not analysed or run through BLAST as it was clear that the bat specific primers designed had worked.

3.4 Sequence Analysis

All sequences obtained were loaded into Geneious and analysed manually. Short or messy sequences that had clearly been unsuccessful, were discarded. All remaining sequences were searched for the appropriate primer. In total, the correct primer was identified and removed from 12 sequences. All sequences were run through BLAST[3] to check that the sequences returned top hits to bat sequences held in the database and in particular *M. tuberculata*.

None of the specimens sampled were successfully able to be sequenced for all four primers. This means that sequences were not obtained for paired primers (i.e. both Myst424F and HBat15958R or Myst346F and Myst408R) for any of the specimens. Separate alignments were therefore created for each primer (given it does not make logical sense to pair alignments from different specimens, which are likely to be different individuals). Alignments were named as follows:

- Alignment R1 – Myst424F
- Alignment R2 - HBat15958R
- Alignment R3 – Myst346F
- Alignment R4 – Myst408R.

The 232 *M. tuberculata* control region sequences used to design the primers were also added to the consensus alignment to ensure that the sequences were aligned correctly. The sequences were then edited by eye to ensure the correct bases had been assigned.

The HBat15958R sits outside the region of the 232 *M. tuberculata* alignment because this was the reverse primer used to obtain these sequences. The full *M. tuberculata* genome (AY960981.1) was therefore used to align the sequences obtained using primer Myst_424F (the forward primer paired with

[3] Basic Local Alignment Search Tool
https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=Betacoronavirus&utm_campaign=wuhan_nCoV&utm_source=insights&utm_medium=referral

HBat15958R). Alignment R1 (Myst_424F) therefore aligns ~30 bp beyond the 232 *M. tuberculata* control sequences used to align the other three alignments.

3.5 Phylogenetic Trees

A phylogenetic tree was created for each of the alignments where SNPs were identified (R1 and R4) using the latest version of BEAST (Suchard, et al., 2018) (v1.10.4). Each alignment was imported as a fasta file.

The alignments were run using the Akaike Information Criterion (AIC) scores generated in jModeltest v2.1.7 (Darriba et al., 2012), which were used to identify the best-fit candidate models of nucleotide evolution (HKY for alignment R1 and GTR+I+G for alignment R4). All files were run with a chain length of 10 million. All trees were run through TreeAnnotator with a burnin percentage of 10. Trees were then displayed using FigTree (A. Rambaut, 2018).

To improve readability of the phylogenetic trees created all *M. tuberculata* sequences were collapsed into the evolutionary significant units (ESUs) identified by Lloyd (2003). It should be noted however that while the three ESUs identified (Northland, Central and Southern North Island (CSN) and South Island) can be justified on the basis of current geographical distribution they do not fully explain the component evolutionary lineages (Lloyd B. D., 2003). For the purpose of this thesis the groupings have been used to facilitate the interpretation of patterns given the extensive number of samples included in each consensus alignment.

Chapter Four – Results

4.1 DNA Samples

In total, nine of the 36 samples resulted in successful sequences. A handful of samples were successful for more than one primer, resulting in 12 successful sequences obtained (shown in Appendix D). In total therefore 12 sequences were obtained from nine samples (using the four bat-specific primers). Appendix E provides a summary of the number of samples collected from each specimen number and the total number of successful samples.

Table 2 also summarises the samples analysed, with an indication of which samples were successful for each primer.

Table 2: Summary of samples and successful sequences based on the primer used. Yellow highlight indicates PCRs that generated a band of approximately the correct size during electrophoresis; Orange borders represents where sequences were obtained from samples sent away for sequencing and a 'Y' indicates where these sequences were identified as bat sequences.

		Primer				
Sample	**Location**	**16Smam**	**Myst408R**	**Myst346F**	**Myst424F**	**HBat15958R**
NMNZ LM001511A	'Stewart Island'					
NMNZ LM001511B	'Stewart Island'					
NMNZ LM001511C	'Stewart Island'			Y		
NMNZ LM001512A	'Stewart Island'					
NMNZ LM001512B	'Stewart Island'					
NMNZ LM001512C	'Stewart Island'					
NMNZ LM001512D	'Stewart Island'					
NMNZ LM001513A	'Stewart Island'					
NMNZ LM001513B	'Stewart Island'					
NMNZ LM001270.1A	Wairarapa					
NMNZ LM001270.1B	Wairarapa					
NMNZ LM001270C	Wairapa					
NMNZ LM001270.2	Wairarapa					
NMNZ S.34270	North Canterbury					
NMNZ S.34270B	South Canterbury					
NMNZ S.32399	Tasman					
NMNZ S.32373	Tasman					
NMNZ S.38824	Tasman		Y			
NMNZ S.38824B	Tasman		Y			
NMNZ S.34376	South Canterbury			Y		Y
NMNZ S.34160	South Canterbury		Y		Y	
NMNZ S.34127	South Canterbury					
NMNZ S.33918	South Canterbury					

		Primer				
Sample	**Location**	**16Smam**	**Myst408R**	**Myst346F**	**Myst424F**	**HBat15958R**
NMNZ S.34224	South Canterbury					
NMNZ S.32324	Tasman			Y		
NMNZ S.33699	North Canterbury			Y		
NMNZ S.30161	Tasman			Y	Y	
NMNZ S.39187	Tasman					
NMNZ S.35793	Hawkes Bay					
NMNZ S.35495	Hawkes Bay					
NMNZ S.34237	South Canterbury				Y	
NMNZ LM001891	Solomon Is.					
NMNZ DM1629.1	Big South Cape Is.					
NMNZ DM1629.3	Big South Cape Is.					
NMNZ DM1629.4	Big South Cape Is.					

All successful sequences were run through BLAST using the reference sequence NCBI nucleotide database to determine whether the sequences matched bat DNA, and in particular, *M. tuberculata*.

Table 3 summarises this search. All sequences matched *M. tuberculata* sequences in GenBank – the percentage identical number provided by GenBank is also provided in brackets. Three of the shorter sequences (NMNZ S.34160, NMNZ S.38824 and NMNZ S.38824B) matched 100% to a range of sequences in GenBank, including plants. '*Mystacina'* was added to the search term to confirm whether these sequences also matched *M. tuberculata* sequences. Given that all the sequences associated with primer 408R initially matched other species (including plants) the results from this primer may have limited utility.

Table 3: BLAST (NCBI nucleotide database) search results, including search function used for 'optimise' and percentage match to M. tuberculata for all 12 successful sequences

Sample	**Primer**	**Location**	**BLAST (percentage identical)**
NMNZ S.30161	Myst_424F	Tasman	*M. tuberculata* (98.63%)
NMNZ S.34160	Myst_424F	S. Canterbury	*M. tuberculata* (95.83%)
NMNZ S.34237	Myst_424F	S. Canterbury	*M. tuberculata* (98.61%)
NMNZ S.34376	HBat15958R	S. Canterbury	*M. tuberculata* (98.57%)
NMNZ S.30161	Myst_346F	Tasman	*M. tuberculata* (94.59%)
NMNZ S.32324	Myst_346F	Tasman	*M. tuberculata* (90.62%)
NMNZ S.33699	Myst_346F	N. Canterbury	*M. tuberculata* (91.67%)
NMNZ LM001511C	Myst_346F	Stewart Is.	*M. tuberculata* (96.97%)
NMNZ S.34376	Myst_346F	S. Canterbury	*M. tuberculata* (100%)
NMNZ S.38824	Myst_346F	Tasman	*M. tuberculata (94.44%)*
NMNZ S.34160	Myst_408R	S. Canterbury	*M. tuberculata* (100%)
NMNZ S.38824	Myst_408R	Tasman	*M. tuberculata* (89.66%)

Sample	Primer	Location	BLAST (percentage identical)
NMNZ S.38824B	Myst_408R	Tasman	*M. tuberculata* (89.29%)

4.2 Analysis

A total of 12 successful sequences from nine different specimen groups were produced. **Error! R eference source not found.** below provides more detail on the location each sample was found.

Table 4: Detailed location information for successful samples

Sample	Collection date	Location
NMNZ S.30161	Aug-1992	Kairuru Cave, Takaka Hill- Entrance Zone
NMNZ S.34160	Apr-1994	Ledge cave, Glenlea Station, South Canterbury
NMNZ S.34237	Feb-1994	Gordons Valley Stn; South Canterbury; Site 3
NMNZ S.34376	Nov-1993	Site 1, Hanging Rock, South Canterbury
NMNZ S.32324	Aug-1991	Hawkes Cave, Takaka Hill Owl pellet material (*Sceloglaux*)
NMNZ S.33699	Sep-1993	Ardenest, Laughing owl nest site, Waikari, North Canterbury
NMNZ S.34160	Apr-1994	Ledge cave, Glenlea Station, South Canterbury
NMNZ S.38824	Jun-1999	Resurgence GD 101 Cave. Gouland Downs. Owl site.
NMNZ LM001511	Jun-1951	'Stewart Island'

When comparing the 12 sequences obtained during this thesis with *M. tuberculata* sequences obtained by Brian Lloyd, clear differences can be observed in a number of sequences. This supports the conclusion that *M. robusta* is a separate species to *M. tuberculata*.

Alignment R1 includes only one *M. tuberculata* sequence as there was only one sequence (AY960981.1) available on Genebank which covered the correct location. In total, for alignment R1 (Myst_424F), 4 single nucleotide polymorphisms (SNPs) can be distinguished between the sequences from this study when compared to the *M. tuberculata* sequence (although it is acknowledge that the *M. robusta* sequences were only able to be compare to one *M. tuberculata* sequence).

Table 5: SNP differences between M. tuberculata *and specimens of* M. robusta *with positions relative to reference sequence AY960981.1*

Sequence	552	553	557	568
M. tuberculata	C	A	C	G
NMNZ S.30161 (Tasman)	**T**	A	C	G
NMNZ S.34160 (S. Canterbury)	C	**G**	**T**	**T**
NMNZ S.34237 (S. Canterbury)	C	**G**	C	G

In addition, to the SNPs noted between the *M. robusta* sequences and the *M. tuberculata* sequences, in two locations (552 and 553) the bases shown differed between the Tasman and South Canterbury *M. robusta* sequences.

Alignment R2 (HBat15958R) included one successful sequence extracted as part of this thesis, sequence NMNZ S.34376. This sequence appears to be very similar to the *M. tuberculata* sequences with only one base which differs from the rest of the sequences (an adenine rather than a guanine at position 477).

Alignment R3 (Myst_346F) contained the most sequences that were successful, however all the sequences were reasonably short.

The sample NMNZ S.34376 is also included in this consensus sequence again because this sample was successfully sequenced using both the HBat15958R and Myst_346F primers. No SNPs were observed between this sequence and the *M. tuberculata* specimens, reinforcing the supposition that the bone this sample was sequenced from actually belonged to a *M. tuberculata*.

No obvious differences (i.e. all the bases can also be observed in several of the *M. tuberculata* sequences) were noted for NMNZ S.30161, NMNZ S.32324, NMNZ S.33699, NMNZ S.38824, or NMNZ LM001511C.

Alignment R4 (Myst_408R) contained some very short sequences, despite this there are enough similarities between the *M. tuberculata* specimens to indicate that the sequences are of bat DNA. No unique SNPs were identified for this alignment, however in several places the *M. robusta* sequence base only occurred very rarely (<2%) in the *M. tuberculata* sequences. Table 6 below summarises these locations.

Table 6: SNP differences between M. tuberculata and specimens of M. robusta with positions relative to reference sequence AY197070.1. Note the numbers in brackets denote the small number of M. tuberculata *sequences that matched the* M. robusta *base*

Sequence	**354**	**357**	**359**	**368**
M. tuberculata	A (4)	C (4)	T (1)	A (1)
NMNZ S.34160 (S. Canterbury)	**G**	**A**	-	-
NMNZ S.38824 (Tasman)	**G**	**A**	T	**G**
NMNZ S.38824B (Tasman)	**G**	**A**	**C**	A

Note: numbers in brackets denote the small number of M. tuberculata *sequences that matched the* M. robusta *base*

4.2 Phylogenetic Trees

Given that no sample was successful for paired primers (i.e. both Myst424F and HBat15958R or Myst346F and Myst408R), four consensus sequences were created, one for each of the four primers.

- Alignment R1 – Myst424F
- Alignment R2 - HBat15958R
- Alignment R3 – Myst346F
- Alignment R4 – Myst408R

A phylogenetic tree was created for the two alignments where SNPs were observed. As shown in Figure 3and Figure 4 (refer to Appendix F for larger versions).

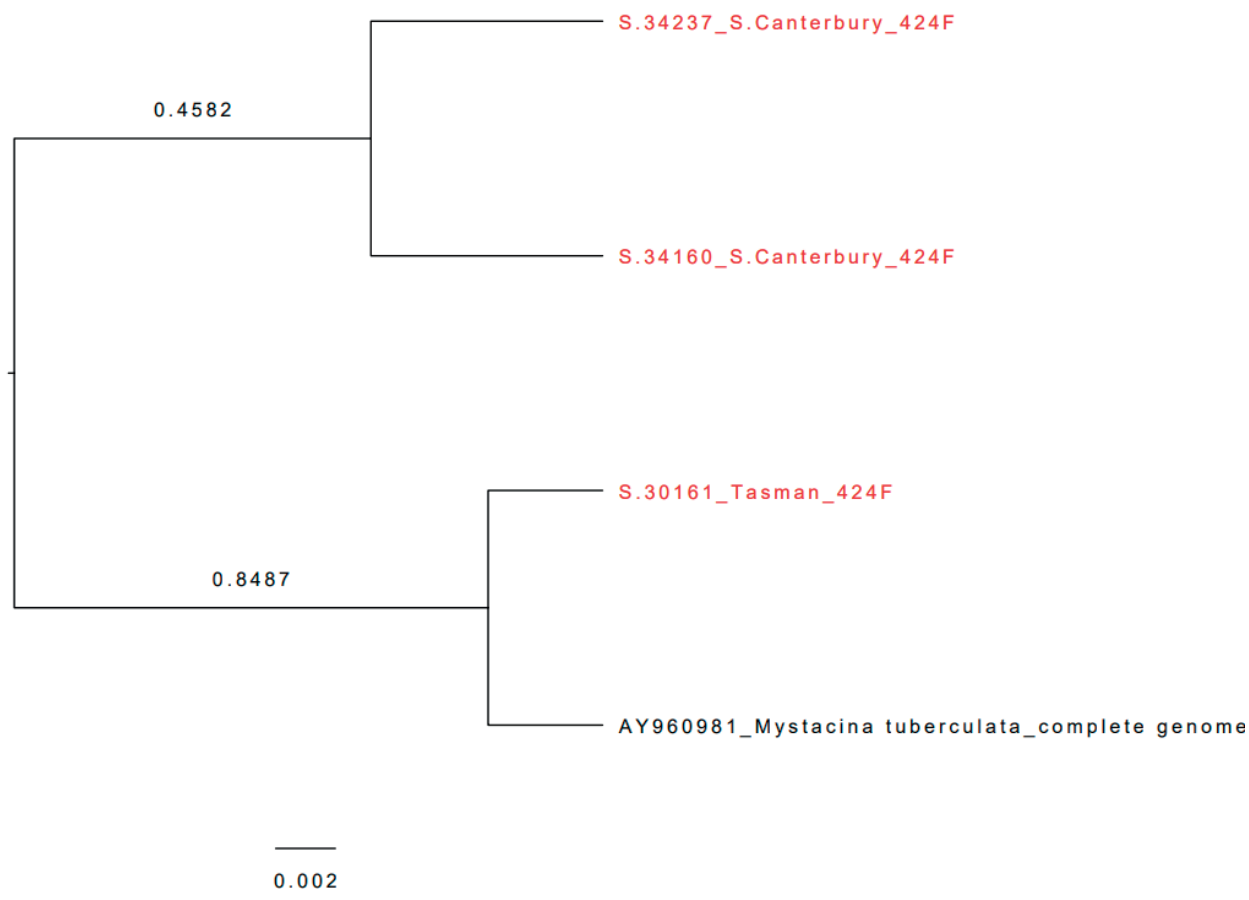

Figure 3: Consensus alignment R1, phylogenetic tree showing evolutionary significant units for M. tuberculata *sequences. Run using the AIC scores generated in jModeltest v2.1.7 (Darriba et al., 2012), which were used to identify the best-fit candidate model of nucleotide evolution (HKY). Posterior probability values shown on tree branches.*

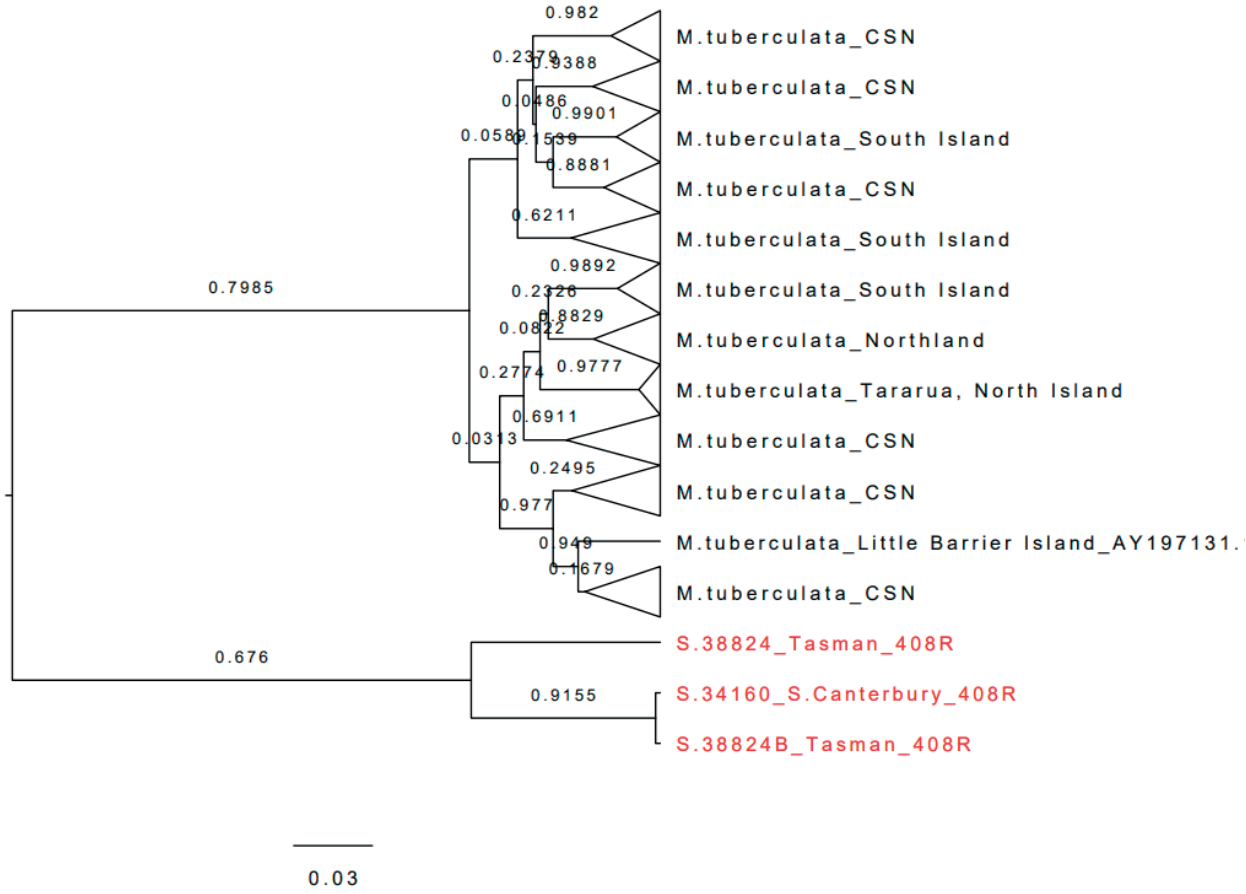

Figure 4: Consensus alignment R4, phylogenetic tree showing evolutionary significant units for M. tuberculata *sequences. Run using the AIC scores generated in jModeltest v2.1.7 (Darriba et al., 2012), which were used to identify the best-fit candidate model of nucleotide evolution (GTR+I+G). Posterior probability values shown on tree branches.*

Alignment R1 (Figure 3) shows both South Canterbury specimens (NMNZ S.34237 and NMNZ S.34160) as distinct species to the *M. tuberculata* sequence. In contrast the Tasman specimen (NMNZ S.30161) fell within the *M. tuberculata* clade. For alignment R4 all of the three specimens (NMNZ S.38824, NMNZ

S.38824B and NMNZ S.34160) fell as distinct specimens. Specimen NMNZ S.34160 therefore showed as a distinct species in both alignments R1 and R4 (the only two alignments this specimen was included in).

Chapter Five – Discussion

5.1 Method

The modified soaking method was successfully used to extract DNA from *M. robusta* bones. All four primers used resulted in at least one successful sequence, with primer Myst_346F resulting in the greatest number of successful sequences. While 12 successful sequences is a small proportion of the total samples run, a small success rate is not unexpected for ancient DNA given the likely degraded state of the DNA and the potential for cross contamination. The results indicate that this method and the primers used are able to be used to sample further specimens to build a more robust picture of the genetic diversity between geographic regions.

In general the tradition Qiagen kit methodology was unsuccessful for many of the more recent specimens, (specimens which died and were collected in the 1950s or 60s). This includes specimens NMNZ LM001511, NMNZ LM001512, NMNZ LM001513 samples and NMNZ DM1629 specimens. Only one successful sequence was obtained from NMNZ LM001511 – NMNZ LM001511C. It is not clear why the method used in this thesis was less successful for the more recent samples.

The NMNZ DM1629 samples, while currently preserved in ethanol, given their age may once have been preserved in formaldehyde. While there is no evidence of this occurring it is understood that at the time these specimens were collected and preserved, the common preservation agent was formaldehyde. Formaldehyde degrades the DNA and makes extracting DNA sequences much harder (Srinivasan, Sedmak, & Jewell, 2002; Zimmermann, et al., 2008). If further sampling was undertaken in the future therefore it may be better to focus on the NMNZ LM001511, NMNZ LM001512 and NMNZ LM001513 specimens and in particular NMNZ LM001511.

5.2 DNA Sequences

Alignment R1 included 4 SNPs between the specimens sequenced for this thesis and the *M. tuberculata* sequence . In addition two of the sequences (NMNZ S.34237 and NMNZ 3.4160) fell within a separate clade to the *M.tuberculata* sequence. This appears to support the hypothesis that the specimens sampled are from *M. robusta* and that *M. robusta* is a distinct species from *M. tuberculata*, although it is noted that the *M. robusta* sequences were only able to be compared against one *M. tuberculata* sequence due to their location. Some minor geographic differences were also observed between

sequence NMNZ S.30161 from the Tasman region (which fell within the same clade as *M. tuberculata*) and the two Canterbury samples.

The specimen NMNZ S.34376 was successfully sequenced using both primer Myst_346F and primer HBat15958R. The first sequence showed no SNPs when compared to *M. tuberculata* specimens (alignment R3) and only one in alignment R2 (an adenine rather than a guanine at position 477). The most common damage-derived errors in ancient DNA is the hydrolytic deamination of cytosine into uracil leading to an apparent change of the base from C to T or substitutions in the DNA sequenced after PCR from a G to an A (Rizzi, Lari, Gigli, De Bellis, & Caramelli, 2012). It is possible that this second error has occurred in the above sequence.

The bone that is sample NMNZ S.34376 is most likely from a *M. tuberculata*, rather than a *M. robusta*. Given that this was the only successful sequence for primer HBat15958R it is therefore not clear whether this primer would be an effective primer to use on *M. robusta* samples.

A further five specimens are included in the R3 alignment, no SNPs were identified for any of these specimens. It should be noted also that alignment R3 had the most variation amongst the *M. tuberculata* specimens, this makes it less likely that SNPs will be identified for any *M. robusta* specimens sequenced. Two of these five specimens were also included in other alignments (NMNZ S.30161 in R1 and NMNZ S.38824 in R4). Specimen NMNZ S.30161 only included one SNP in alignment R1 and none in alignment R3 and therefore it is unclear what species this specimen could be attributed. Specimen NMNZ S.38824 included three (rare, but not unique) SNPs in alignment R4 and fell as a distinct species from *M. tuberculata* in the phylogenetic tree for alignment R4. This suggests that this specimen may be *M. robusta*. The remaining three specimens (NMNZ S.32324, NMNZ S.33699 and NMNZ LM01511C) were only included in alignment R3 and therefore it is unclear whether these specimens are *M. tuberculata* or *M. robusta*. While no SNPs were identified for these three specimens, as noted this may be due to the short sequence length and variation within the *M. tuberculata* specimens. Further analysis would be required to determine which species these specimens should be attributed to.

No unique SNPs were identified for alignment R4, however a number of bases only occurred rarely (<2%) in the *M. tuberculata* sequences. Specimen NMNZ S.34160 showed two rare SNPs and NMNZ S.38824 and NMNZ S.38824B showed three rare SNPs.

No further geographic differences were observed between any of the specimens sequenced in this thesis beyond what has been discussed for alignment R1 above.

5.3 Phylogenetic Trees

Two phylogenetic trees were produced from the two consensus alignments where SNPs were identified. These trees are included in Appendix F.

In the alignment R1 phylogenetic tree two specimens fell as distinct species (NMNZ S.34237 and NMNZ S.34160), while specimen NMNZ S.30161 fell with the clade containing *M. tuberculata* specimens.

All three specimens from this thesis included in the R4 alignment fell as a distinct species to the *M. tuberculata* specimens in the R4 phylogenetic tree. In addition the posterior values indicated a reasonable degree of confidence in these placings.

5.4 Conclusion

This thesis has successfully demonstrated that DNA can be extracted from ancient museum bone specimens, and in particular very small bones, without damaging the sample. No other study has successfully extracted DNA from, and obtained sequences, for *M. robusta*. This study extracted 12 sequences from nine specimens.

It is considered likely that one specimen sampled (NMNZ S.334376 Canterbury) is *M. tuberculata.* Sequences from four specimens (NMNZ S.34160, NMNZ S.34237, NMNZ S.38824 and NMNZ S.38824B) differed by multiple SNPs from >200 existing *M.* tuberculata sequences, strong suggesting that they are *M. robusta*. It is unclear which species the remaining specimens may be. Further analysis would be required for all specimens to provide more clarity on the likely species.

While some evidence of difference across different geographic regions may have been observed in this study, there are too few sequences available to be confident that this is the case. It is interesting to note that Lloyd (2003) found haplotypes of *M. tuberculata* were distributed across wide areas of New Zealand, but when the frequency of the haplotypes were assessed difference between regions could be identified. It is possible that a similar haplotype distribution occurred in *M. robusta*.

Now that a non-destructive method of DNA analysis has been shown to be possible for this taxon, further extractions can be undertaken across a wider range of specimens to identify possible genetic diversity between regions prior to human arrival.

It is hoped that these sequences may assist with future conservation efforts should further evidence of *M. robusta's* continued existence be discovered. In particular now that sequences of *M. robusta* specimens are available these can be compared to any environmental DNA or bat DNA which may be collected in the future.

Further work to sample New Zealand bat specimens from more recent samples would also be beneficial if sufficient sequences could be obtained to draw conclusions regarding any possible loss of genetic diversity once the populations of *M. robusta* became restricted following human arrival. Better results may be obtained from increasing the number of cycles during PCR and sampling a wider range of the specimens (i.e. samples collected from tissue, hair, bones etc.).

Further work to expand the number of *M. robusta* specimens sequence will also provide a more robust dataset which may enable conclusions to be drawn in the future regarding genetic difference across geographic regions of New Zealand. Now *M. robusta* sequences are available future projects may be able to use these sequences to design *M. robusta* specific primers which may result in a greater rate of successful amplifications.

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Appendix A: Te Papa *M. robusta* Specimens Requested for Sampling

Museum #	Locality	Date Collected
NMNZ S.030161	Kairuru Cave, Takaka Hill- Entrance Zone	10 Aug 1992
NMNZ S.032324	Hawkes Cave, Takaka Hill Owl pellet material (Sceloglaux)	01 Aug 1991
NMNZ S.032346	Black Layer, Predator Cave Takaka Hill	1992
NMNZ S.032373	Brown Layer, Predator Cave, Takaka Hill Owl pellet material (Sceloglaux)	1992
NMNZ S.032399	Upper White Layer, Predator Cave, Takaka Hill Owl pellet material (Sceloglaux)	1992
NMNZ S.032416	Lower White Layer, Predator Cave Takaka Hill owl pellet material (Sceloglaux)	1992
NMNZ S.032664	Winter Cave, Takaka Hill	24 Aug 1992
NMNZ S.032693	East Tomo Hawkes Cave Takaka Hill	24 Mar 1991
NMNZ S.032694	East Tomo Hawkes Cave Takaka Hill	24 Mar 1991
NMNZ S.032695	East Tomo Hawkes Cave Takaka Hill	24 Mar 1991
NMNZ S.033458	Gowan Hills Owl site; Weka Pass; North Canterbury	07 Oct 1992
NMNZ S.033599	Cave predator site (falcon) Annandale Stn North Canterbury	28 Mar 1993
NMNZ S.033666	Arden Rockshelter 3 Waikari, North Canterbury	27 Sep 1993
NMNZ S.033699	Ardenest, Laughing owl nest site, Waikari, North Canterbury	28 Sep 1993
NMNZ S.033818	Gowan Hill, North Canterbury	04 Sep 1994
NMNZ S.033918	Braeburn Station, South Canterbury	09 Apr 1994
NMNZ S.033987	Sterndale Road cliffs, South Canterbury	07 Apr 1994
NMNZ S.034127	Craigmore Stn; South Canterbury	03 Feb 1994

Museum #	Locality	Date Collected
NMNZ S.034130	Craigmore Stn, South Canterbury, Valley of the Moa	03 Feb 1994
NMNZ S.034160	Ledge cave, Glenlea Station, South Canterbury	06 Apr 1994
NMNZ S.034196	Gordons Valley Stn., South Canterbury; Site 2a	05 Feb 1994
NMNZ S.034224	Gordons Valley Stn; South Canterbury; Site 2b	05 Feb 1994
NMNZ S.034237	Gordons Valley Stn; South Canterbury; Site 3	05 Feb 1994
NMNZ S.034252	Gordons Valley Stn; South Canterbury; Site 4 - upper	05 Feb 1994
NMNZ S.034270	Gordons Valley Stn; South Canterbury; Site 4 - upper	05 Feb 1994
NMNZ S.034303	Site 5, Gordons Valley Station, South Canterbury	05 Feb 1994
NMNZ S.034331	Gordons Valley Stn; South Canterbury Site 7	10 Feb 1994
NMNZ S.034363	Site 8, Gordons Valley Station, South Canterbury	10 Feb 1994
NMNZ S.034376	Site 1, Hanging Rock, South Canterbury	12 Nov 1993
NMNZ S.034397	Site 3, Hanging Rock, South Canterbury	13 Nov 1993
NMNZ S.035495	Deb's Cave, Hukanui, Puketitiri, Hawke's Bay	30 Jan 1997
NMNZ S.035504	Deb's Cave, Hukanui, Puketitiri	30 Jan 1997
NMNZ S.035793	Hakanui 7a (surface) Hawke's Bay	1952
NMNZ S.036484	Hukanui 7a sq 10 spit 6 +	Mar 1998
NMNZ S.036649	Hukanui 7a, Sq. 12, Spit 4, Northern part.	13 Mar 1998
NMNZ S.038667	Ocean Beach, Napier. Loc. 7d (39°42' 3" S, 177°01' 59" E). 200m towards sea from site 7.	14 Sep 1999
NMNZ S.038675	Ocean Beach, Napier. Loc. 8 (39°42' 25.4" S, 177°01' 43.8" E).	10 Dec 1998

Museum #	Locality	Date Collected
NMNZ S.038824	Resurgence GD 101 Cave. Gouland Downs. Owl site.	06 Jun 1999
NMNZ S.039065	Takaka Fossil Cave.	2000
NMNZ S.039187	Takaka Fossil Cave.	2000
NMNZLM001511	Stewart Island	Jun 1951
NMNZ LM001512	Stewart Island	Jun 1951

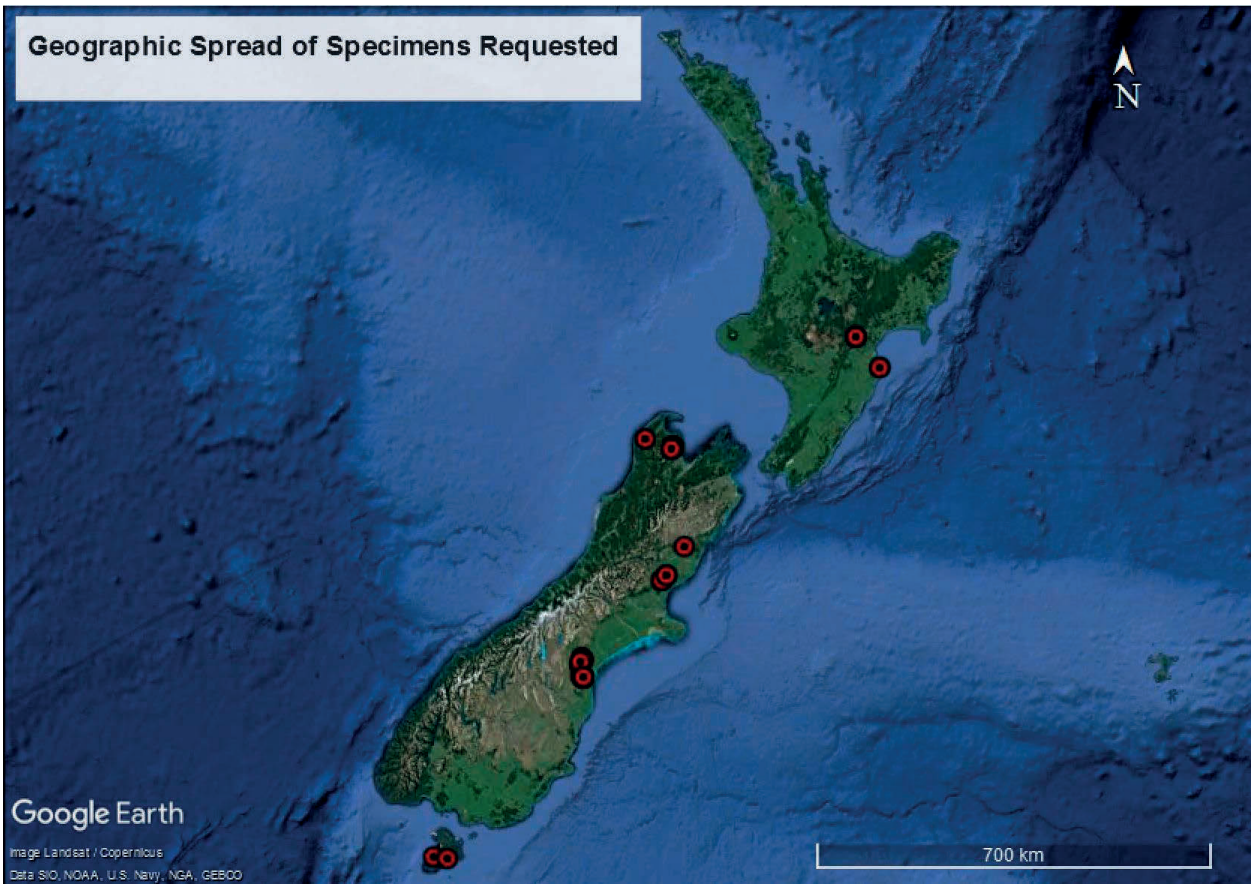


Figure 5: Geographic location of Te Papa specimens requested for sampling as part of this thesis

Appendix B: Soaking Methodology

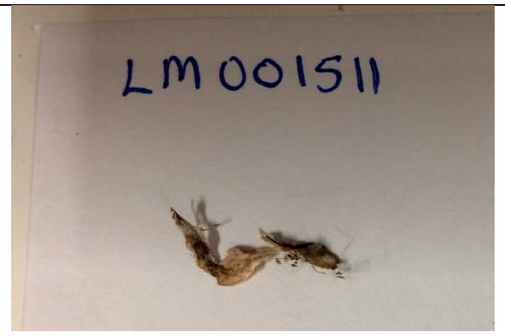
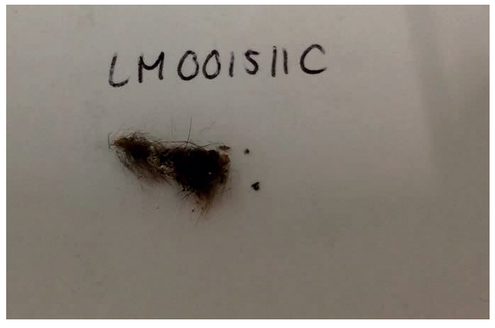
The following steps were undertaken for those samples which used the soaking method. The method adapts the method generally used with a DNeasy blood and bone kit.

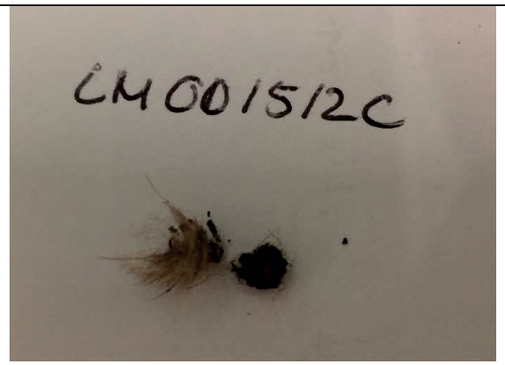
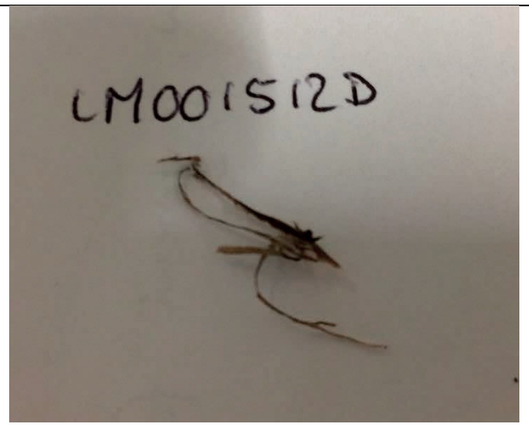
1. Photograph all specimens with labels
2. Pipette 200µL of EDTA, 6µL of SDS and 5µL of protenase K into a tube with the bone specimen
3. Flick the bottom of tube to mix and heat at 45^{o}C in a water bath for between 15 and 35 minutes
4. Remove the bone specimen and place in purified water for at least as long as they were on the heat and then remove and place on labelled paper towels overnight to dry
5. Add 200µL of buffer AL to the resultant solution
6. Vortex
7. Add 200µL 100% ethanol
8. Vortex
9. Pipette all the liquid into a DNeasy column, leaving the dirt behind
10. Spin at 6000g for one minute
11. Discard liquid and use a new tube (note liquid is hazardous)
12. Add 500µL of buffer AW1
13. Spin at 6000g for one minute, discard the liquid and use a new tube (liquid is not hazardous)
14. Add 500µL AW2
15. Spin at 20,000g for three minutes
16. Dry the bottom of the column, tube and liquid can be discarded
17. Add column into a new tube with lid and label on the top of the lid and the side of the tube (date, sample name + DNA)
18. Add 40µL Buffer AE (this needs to be pipetted right into the middle of the column) and leave to sit for at least a minute
19. Spin at 6000g for one minute
20. Add the solution back through the column
21. Spin at 6000g for one minute

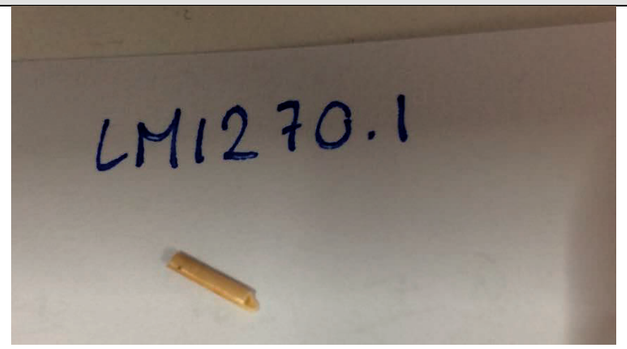
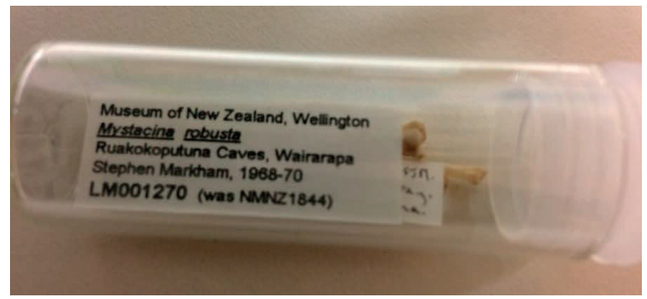
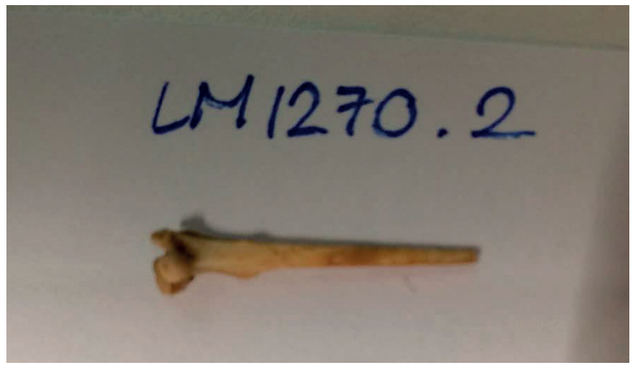
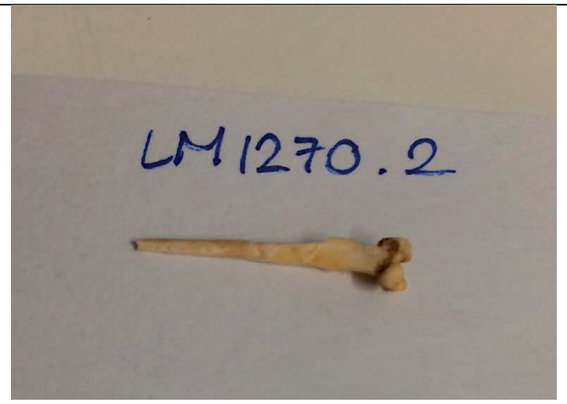
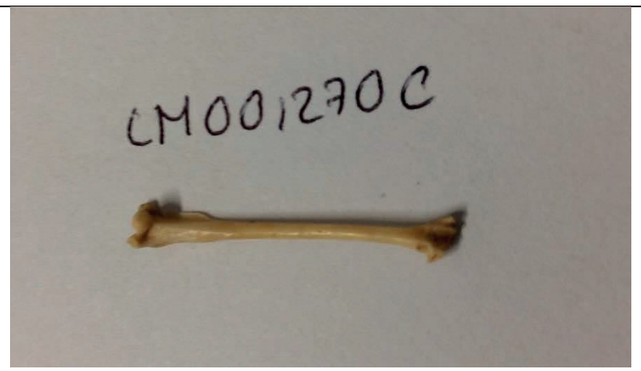
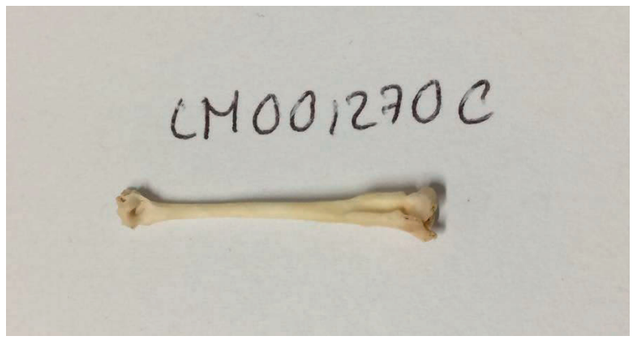
22. Discard the column and keep solution
23. Run the PCR in the modern DNA lab
24. Run on a gel to check which samples worked and then send potentially successful samples away for sequencing.

Appendix C: Photographs of Sample Specimens

(All photographs taken by Isobel Oldfield)

Specimen #	Before extraction	After extraction	Source
NMNZ LM001513		**Not soaked**	
NMNZ LM001511		**Not soaked**	
NMNZ LM001511C		**Not soaked**	

Specimen #	Before extraction	After extraction	Source
NMNZ LM001512		**Not soaked**	
NMNZ LM001512C		**Not soaked**	
NMNZ LM001512D		**Not soaked**	

Specimen #	Before extraction	After extraction	Source
NMNZ LM001270.1		**Not soaked**	
NMNZ LM001270.2			
NMNZ LM001270C			

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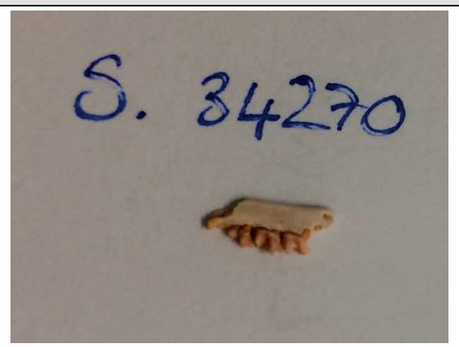
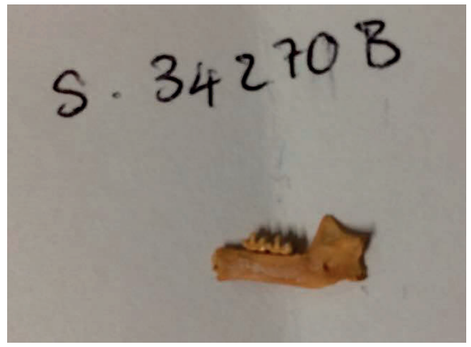
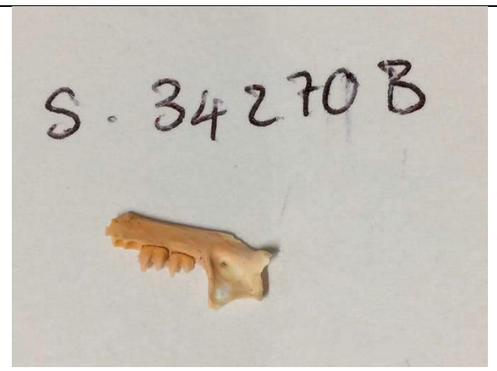
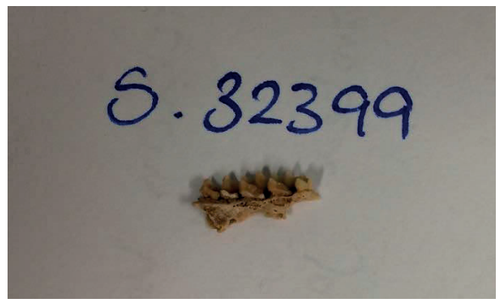
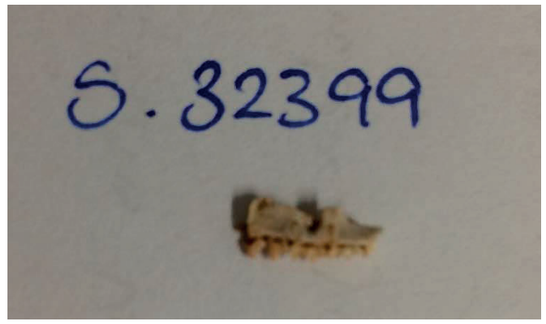
NMNZ S.34270			
NMNZ S.34270B			
NMNZ S.32399			

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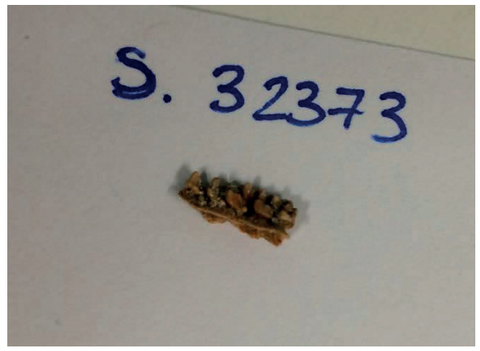
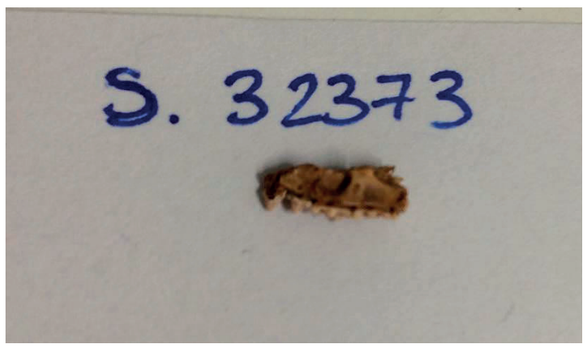
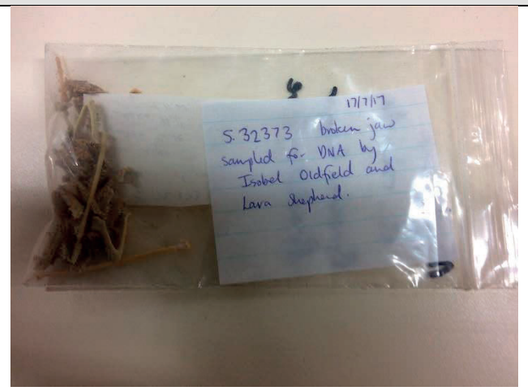
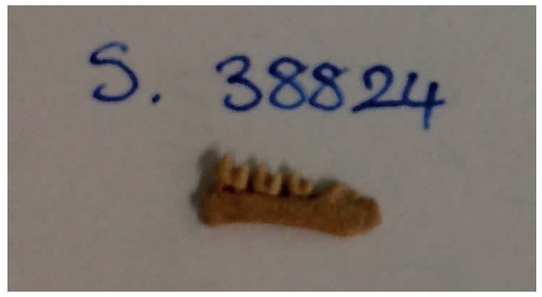
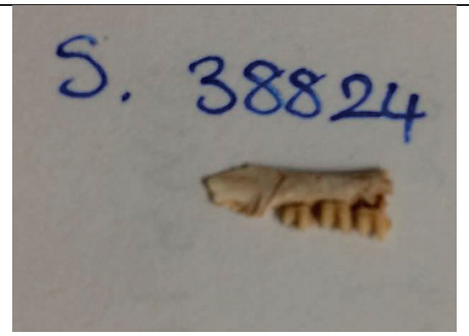
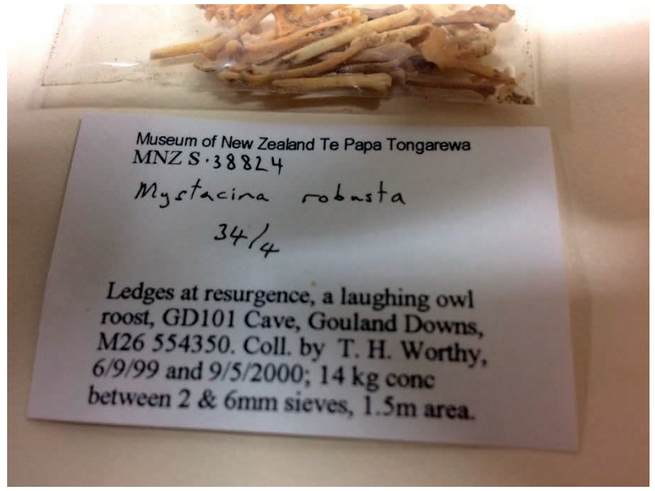
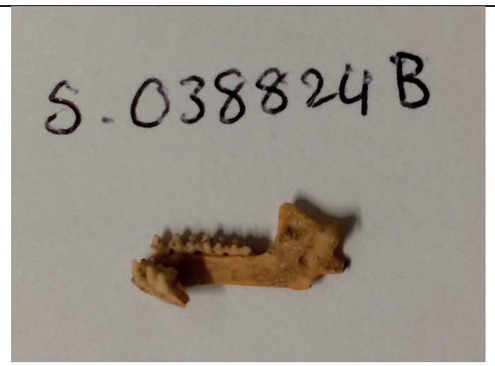
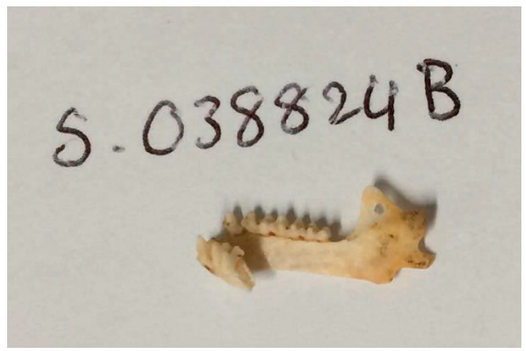
NMNZ S.32373			
NMNZ S.38824			
NMNZ S.38824B			

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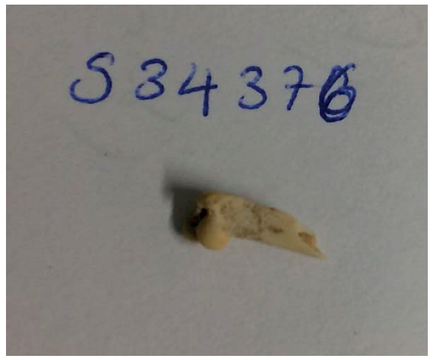
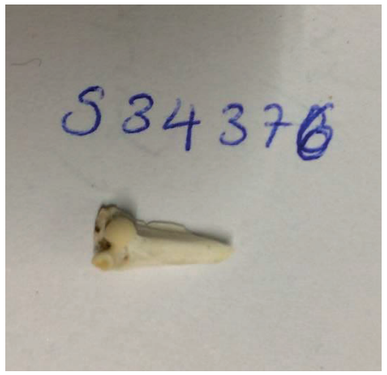
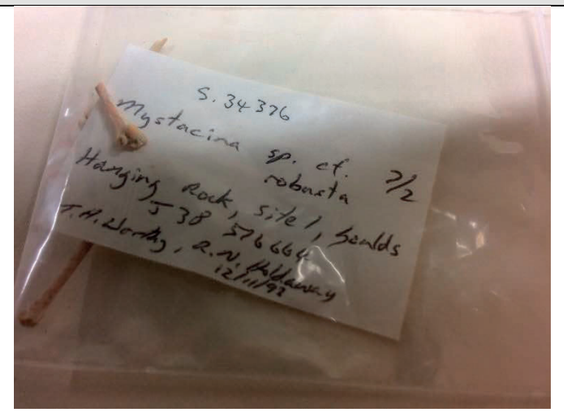
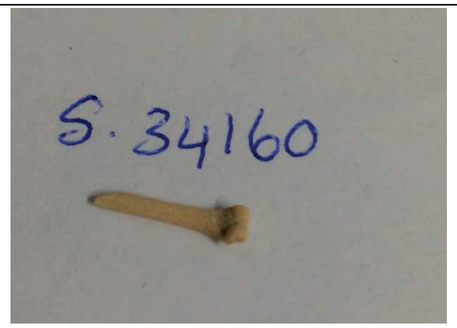
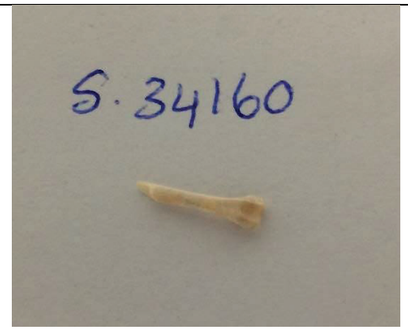
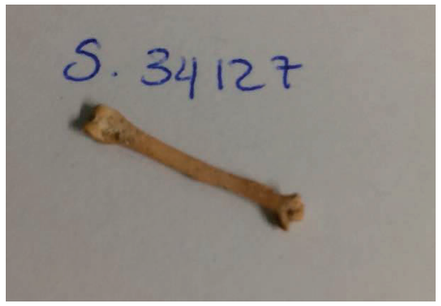
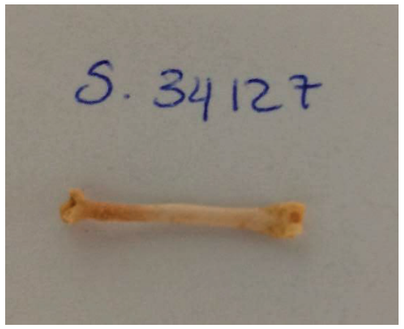
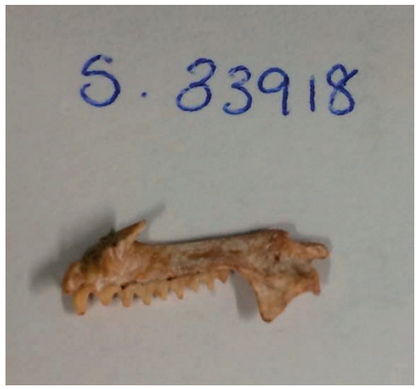
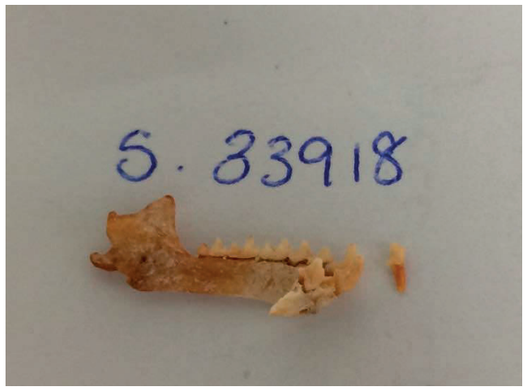
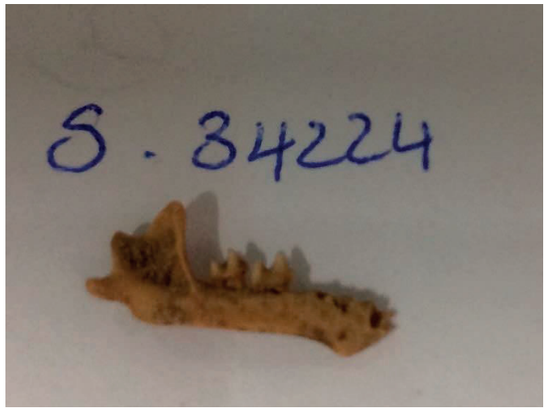
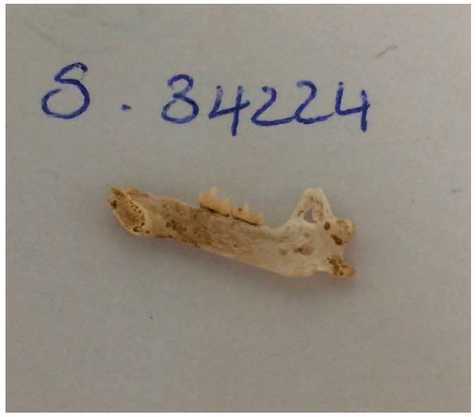
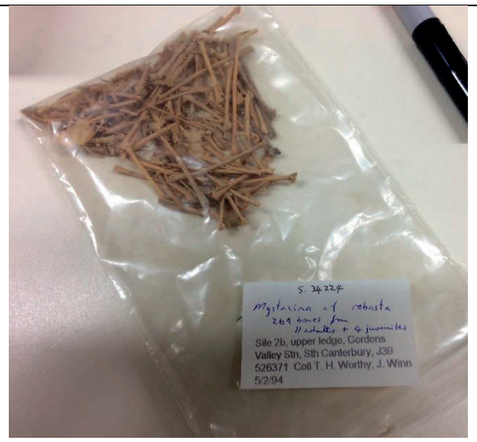
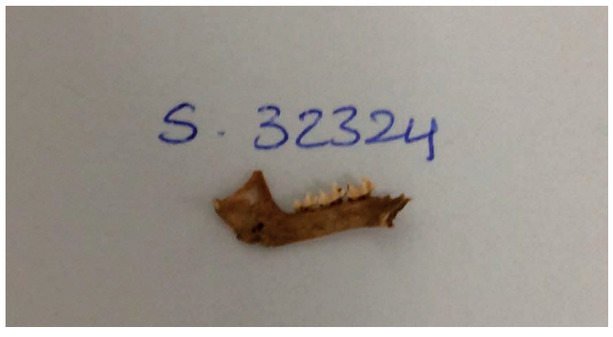
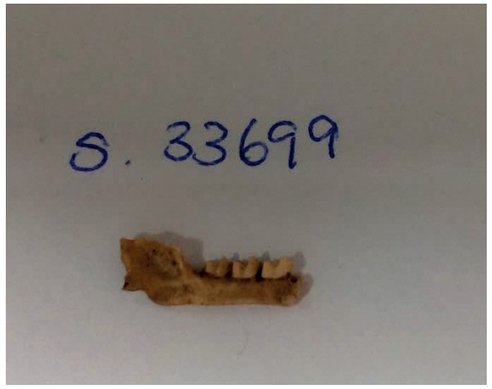
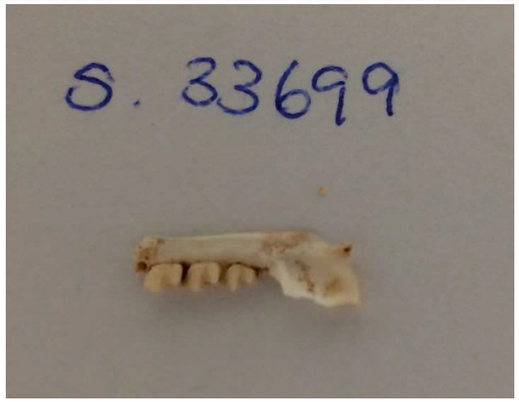
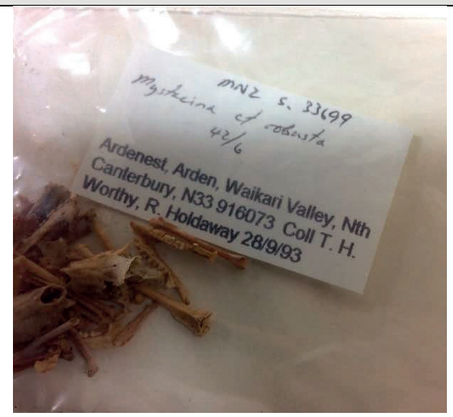
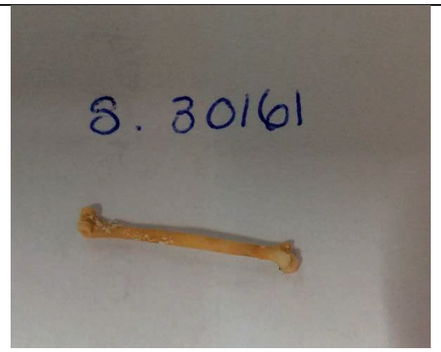
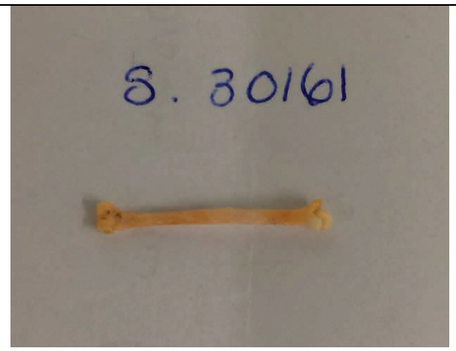
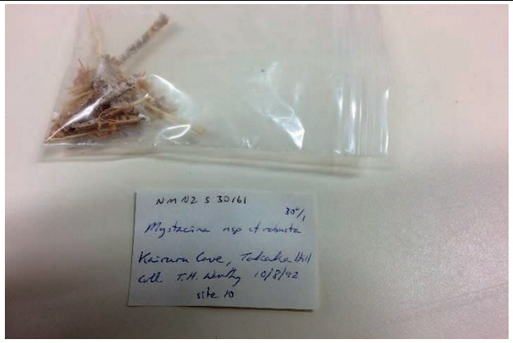
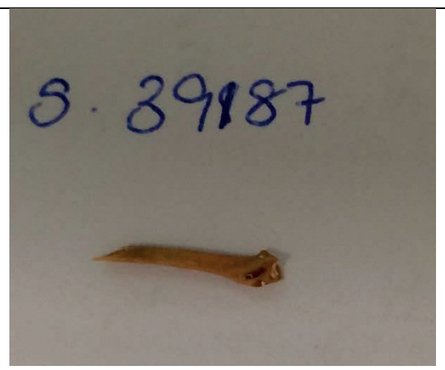
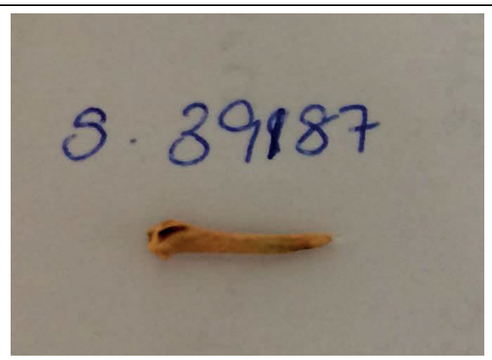
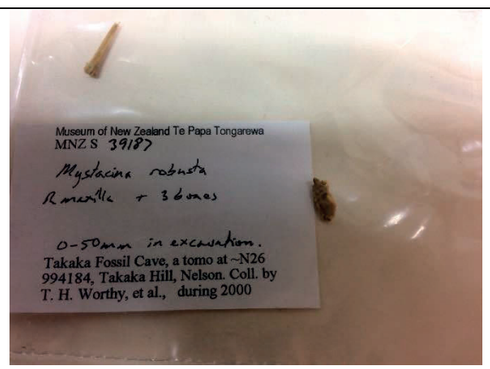
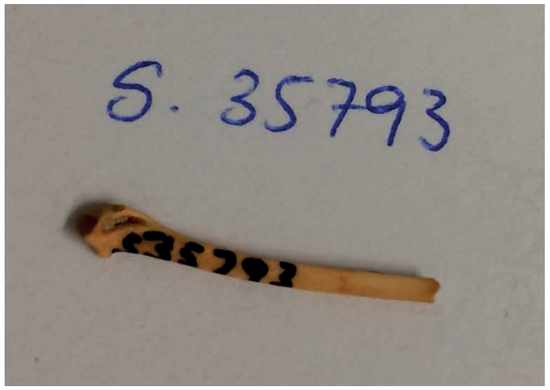
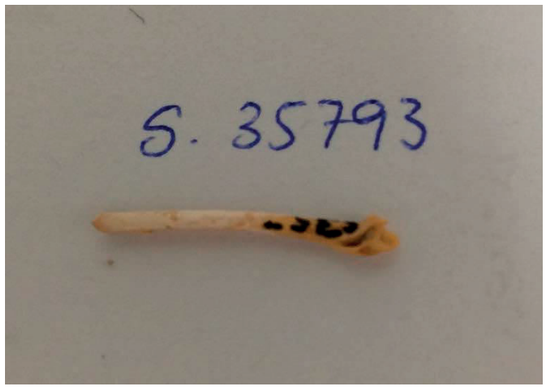
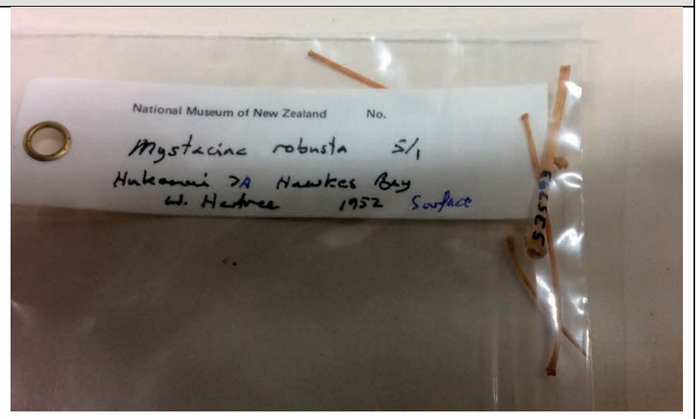
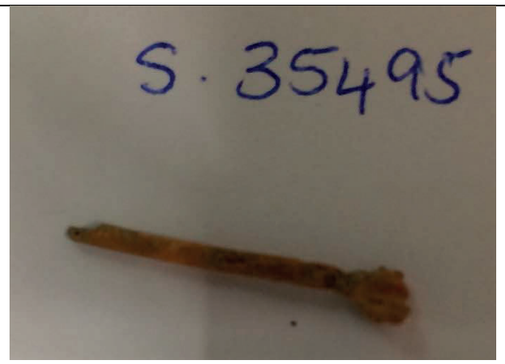
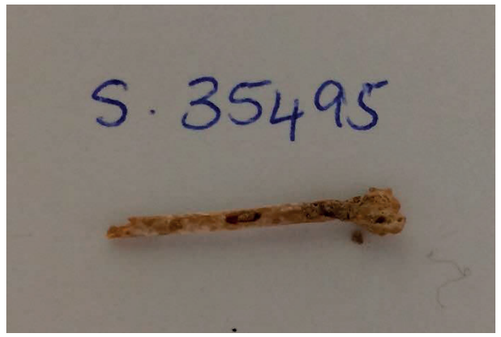
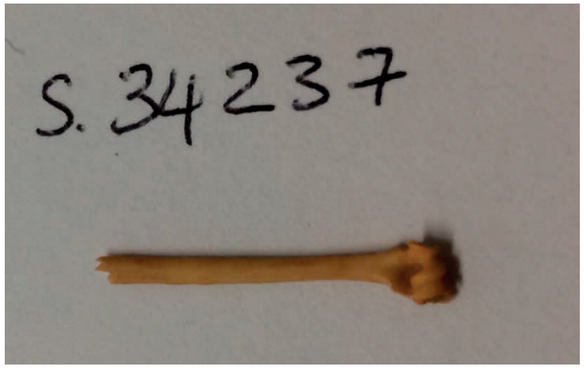
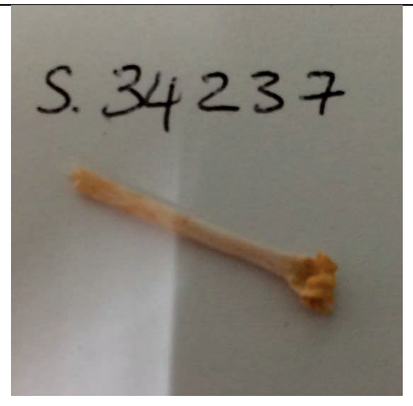
NMNZ S.34376			
NMNZ S.34160			
NMNZ S.34127			

Specimen #	Before extraction	After extraction	Source
NMNZ S.33918			
NMNZ S.34224			
NMNZ S.32324			

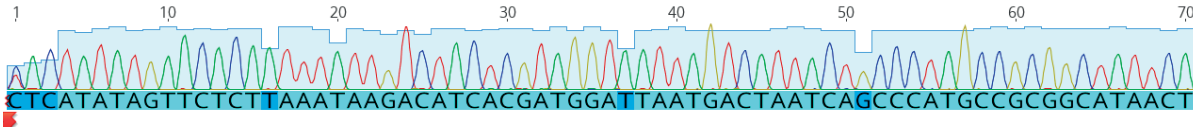
Specimen #	Before extraction	After extraction	Source
NMNZ S.33699			
NMNZ S.30161			
NMNZ S.39187			

Specimen #	Before extraction	After extraction	Source
NMNZ S.35793			
NMNZ S.35495			
NMNZ S.34237			

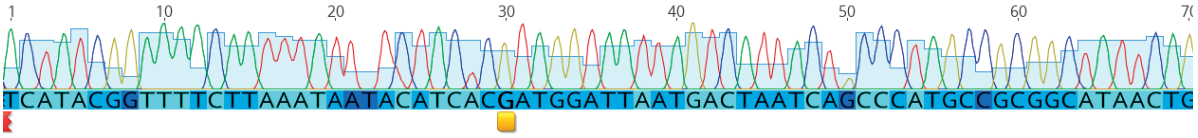
Specimen #	Before extraction	After extraction	Source
NMNZ DM1629[4]	Tissue extracted	Not soaked	
NMNZ LM001891	Tissue extracted	Not soaked	

[4] Note four bats preserved in ethanol fall under this specimen number. Three of the four were sampled. One of those specimens (NMNZ DM1629.1) to be sampled is thought to be a lesser short-tailed bat, rather than a greater short-tailed bat and therefore a sample was taken in the hope of determining whether this was the case.

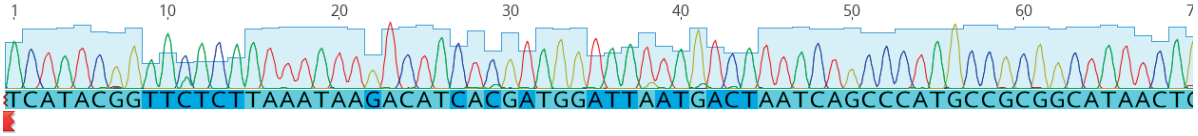
Appendix D: Successful Sequences



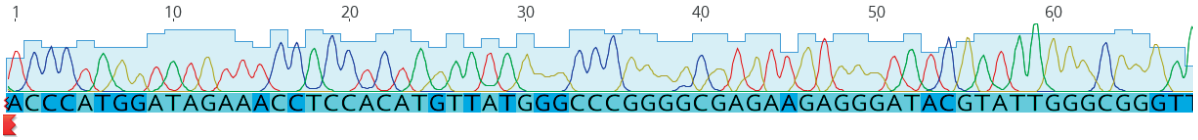
NMNZ S.30161_Tasman_424F



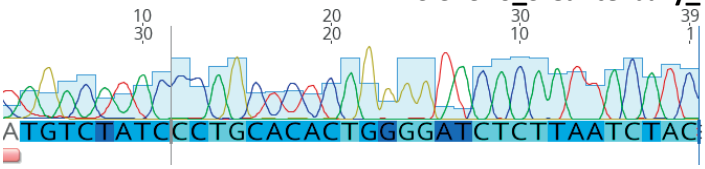
NMNZ S.34160_S.Canterbury_424F



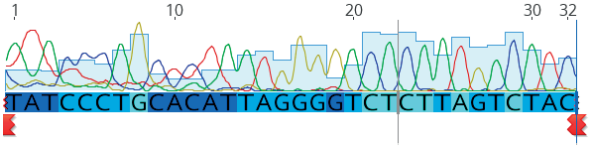
NMNZ S.34237_S.Canterbury.424F



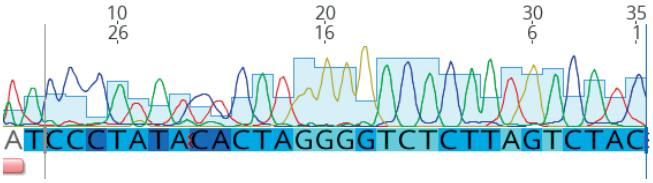
NMNZ S.34376_S.Canterbury_HBat15958R



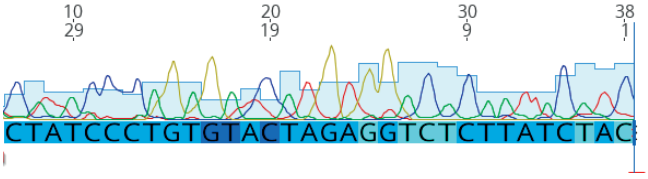
NMNZ S.38824_Tasman_346F



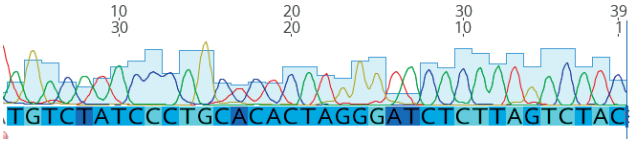
NMNZ S.32324_Tasman_346F



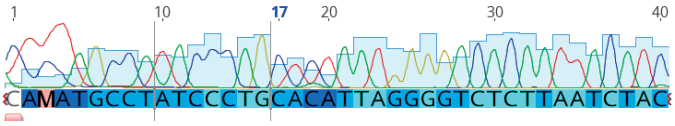
NMNZ S.34376_S.Canterbury_346F



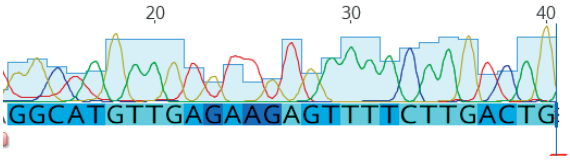
NMNZ LM001511C_Stewart Is._346F



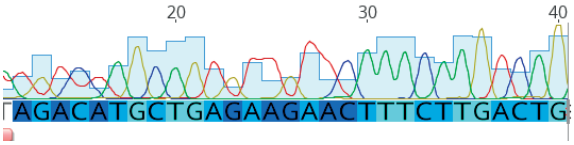
NMNZ S.33699_N.Canterbury_346F



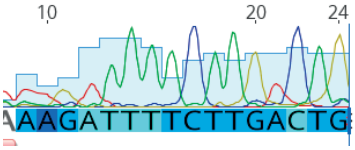
NMNZ S.30161_Tasman_346F



NMNZ S.38824B_Tasman_408R



NMNZ S.38824_Tasman_408R



NMNZ S.34160_S.Canterbury_408R

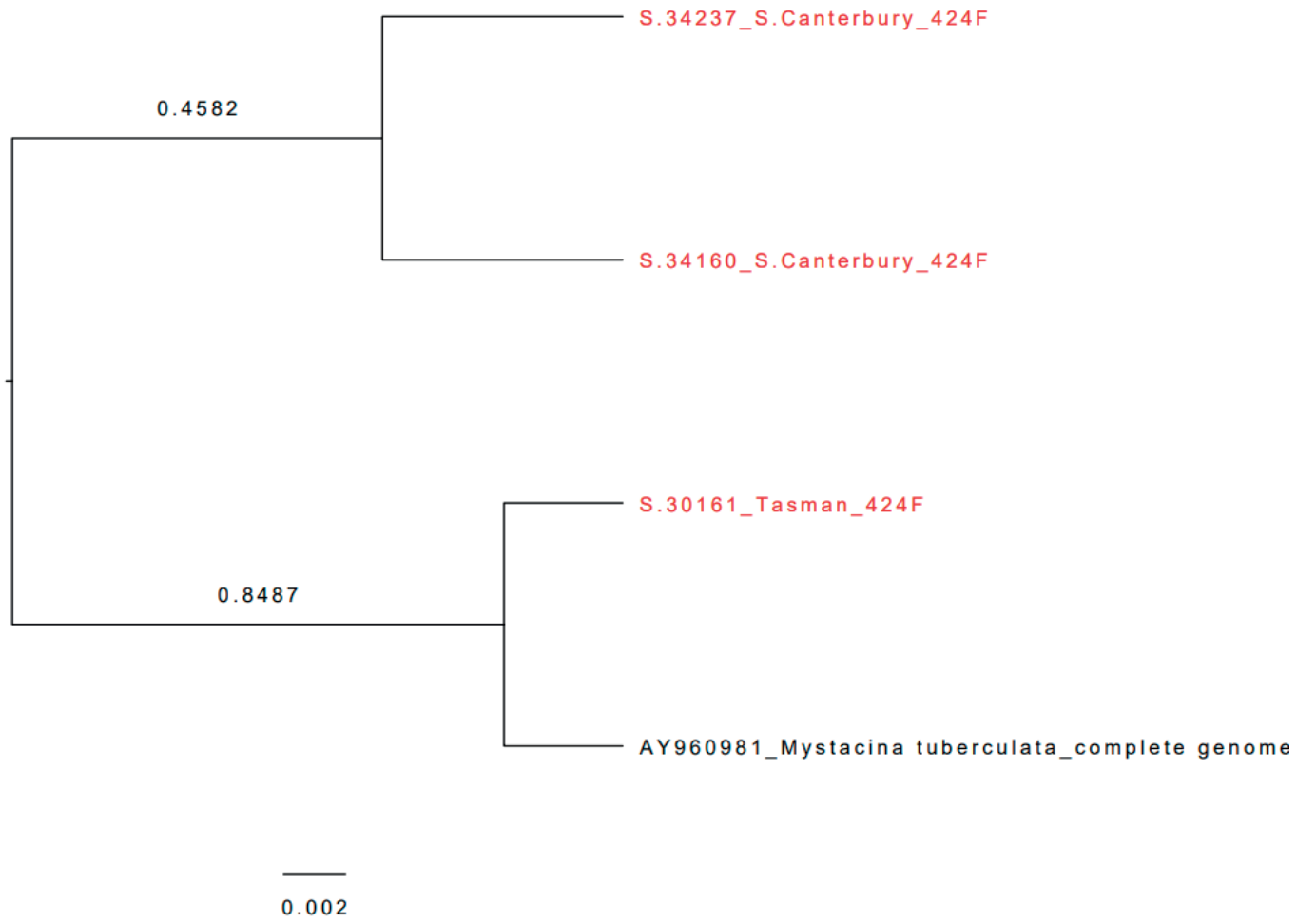
Appendix E: Total Number of Samples and Successful Samples

Specimen #	# Samples (# successful)	Type of Sample	Location
NMNZ LM001511	3 (1)	piece of tissue from ribs	'Stewart Island'
NMNZ LM001512	4	piece of tissue	'Stewart Island'
NMNZ LM001513	2	piece of tissue + fur	'Stewart Island'
NMNZ LM001270	4	bone fragment (dry skeleton)	Wairarapa
NMNZ S.34270	2	broken jaw	North Canterbury
NMNZ S.32399	1	broken jaw	Tasman
NMNZ S.32373	1	broken jaw	Tasman
NMNZ S.38824	2 (2)	broken jaw	Tasman
NMNZ S.34376	1 (1)	bone fragment	South Canterbury
NMNZ S.34160	1 (1)	bone fragment	South Canterbury
NMNZ S.34127	1	whole bone	South Canterbury
NMNZ S.33918	1	broken jaw	South Canterbury
NMNZ S.34224	1	broken jaw	South Canterbury
NMNZ S.32324	1 (1)	broken jaw	Tasman
NMNZ S.33699	1 (1)	broken jaw	North Canterbury
NMNZ S.30161	1 (1)	whole bone	Tasman
NMNZ S.39187	1	bone fragment	Tasman
NMNZ S.35793	1	bone fragment	Hawkes Bay
NMNZ S.35495	1	bone fragment, distal right humerus	Hawkes Bay
NMNZ S.34237	1 (1)	broken humerus	South Canterbury
NMNZ DM1629	4	tissue	Big South Cape
NMNZ LM001891	1	tissue	Solomon Island
Total	36 (9)		

Appendix F: Phylogenetic Trees

Note: all sequences from this thesis are highlighted in red in the following phylogenetic trees.

Alignment R1



Alignment R4